Uptake of hydrophilic solutes through plant leaves: current state of knowledge and perspectives of foliar fertilization

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Abstract

Foliar fertilization is an agricultural practice of increasing importance in practical terms. Since nutrient sprays are mostly applied as water solutions, the focus of the article was placed on the penetration of ionic, polar solutes through the leaf surface, although the mechanisms of cuticular penetration of lipophilics are also taken into consideration. In theory, application of foliar nutrient sprays may indeed be a more target-oriented and environmentally-friendly fertilisation method since nutrients are directly delivered to the target organism in limited amounts, thereby helping to reduce the environmental impact associated with soil fertilisation. However, response to foliar sprays is often variable and not reproducible due to the existing lack of knowledge of many factors related to the penetration of the leaf-applied solution.

To overcome the current "spray and pray" situation, there is a need to critically analyse the major factors involved and the existing experimental approaches to safely assess the penetration mechanisms, which is the final aim of this review. Beginning with the significance of foliar fertilisation in agriculture, a historical overview regarding foliar uptake related studies is given, with especial emphasis on the penetration of solutes through the cuticle and stomata. The existing models of cuticular and stomatal uptake are analysed separately considering among other factors the physico-chemistry of the solution including the role of adjuvants and the effect of the environment. Methods employed to estimate the process of cuticular and stomatal penetration of solutes are critically assessed. Finally, conclusions related to multi-disciplinary research perspectives for improving the efficiency of foliar sprays are drawn.

Keywords cuticle, environmental factors, foliar penetration, foliar sprays, leaf surface, physico-chemistry solution, stomata

I. INTRODUCTION

Plant leaves are organs largely specialized in capturing light and CO_2, which play a critical role in plant survival and productivity (Sinha, 1999). While the basic role of the leaf is strongly conserved (Press, 1999), a great diversity in its morphology, size, surface topography and other features has been observed in the plant kingdom (McLellan, 2005). Such diversity is linked to the water transport capacity of the leaf and some features can be modulated by patterns of water availability during leaf expansion (Zwieniecki *et al.*, 2004; Sack and Holbrook, 2006).

The need for CO_2 uptake for a maximum of photosynthetic gain in a mostly water under-saturated atmosphere resulted in an evolutionary adaption of leaf anatomy and the development of resistances against uncontrolled water losses. These comprise the cuticle, covering all primary aerial surfaces of vascular plants as a non-living, hydrophobic membrane, and stomata as adjustable apertures for the uptake of CO_2. Both adaptations are responsible for the more or less pronounced barrier properties of leaf surfaces not only against the loss of water but also against the penetration of solutes.

The penetration of substances into leaves is a passive process driven by concentration gradients. However, the mechanism is often referred to with the misleading term 'uptake', which, however, does not imply the occurrence of active processes in the penetration through the cuticle (Eichert and Goldbach, 2008). As this

term is widely used in the existing studies, it will also be applied in the present review to describe the penetration of solutes through leaf surfaces.

Nowadays, foliar fertilization has an increasing importance, but the underlying mechanisms controlling the penetration of solutes are not fully understood. Most of the foliar-applied nutrients are ions or polar molecules, and especially for these types of solutes, current knowledge of foliar penetration processes is still very limited. This review summarizes the present status of and identifies the gaps in this research.

II. PRACTICAL SIGNIFICANCE OF FOLIAR FERTILIZATION

In revising the existing literature, a fair amount of foliar fertilisation studies can be found both for macro- and micronutrient sprays and different plant species. At a first glance, it is possible to note the enormous plant response variability and limited reproducibility of results associated with foliar spray treatment. With a few exceptions, the lack of detailed information concerning factors such as the prevailing environmental conditions at the time of treatment, the physical-chemical properties of solutions (e.g., pH, molecular weight or concentration of active ingredients and adjuvants) or the mode of application pose obstacles to interpret the results, thereby supporting the popular saying in the foliar spray scene of "spray and pray". Nevertheless, the use of foliar nutrient sprays in agriculture is increasingly widespread, since they are potentially more environmentally-friendly and target-oriented as compared to root treatments. Currently, foliar fertilisers are commonly supplied to commercial fruit and vegetable crops (Zhang and Brown, 1999a,b).

Research on foliar fertilisation with entire plants under field conditions as developed in the USA since the beginning of the last century enabled the gradual use of foliar nutrient sprays in agriculture. Early foliar spray field studies were chiefly carried out with fruit species (e.g., grapevine, citrus, apple or peach trees) and assessed the effect of iron (Wallace, 1928; Burke, 1932; Guest and Chapman, 1949; Wallihan *et al.*, 1964), magnesium (Boyton, 1945; Walker and Fischer, 1957; Embleton and Jones, 1958), boron (Burrell, 1958), phosphorous (Silberstein and Wittwer, 1951; Eggert and Kardos, 1954; Fisher and Walker, 1954), nitrogen (Hamilton *et al.*, 1942; Weinberger *et al.*, 1949; Bullock *et al.*, 1952; Cook and Boyton, 1952) or zinc and manganese sprays (McClung, 1954; Wallihan and Heymann-Herschberg, 1956; Cook, 1957; Leyden and Toth, 1960; Labanauskas *et al.*, 1963; Embleton *et al.*, 1964; Labanauskas and Puffer, 1964).

A direct relationship between appropriate timing in relation to plant phenology and effectiveness of foliar sprays has been observed for several crops (Alexander, 1986; Weinbaum, 1988; Southwick *et al.*, 1996; Lovatt, 1999). The effectiveness of a nutrient spray is normally assessed in relation to its penetration and availability rate (Lea-Cox and Syvertsen, 1995; Picchioni *et al.*, 1995; Zhang and Brown, 1999a,b; Fernández *et al.*, 2005; Papadakis *et al.*, 2007; Restrepo-Díaz *et al.*, 2008; Val *et al.*, 2008), reduced phyto-toxicity (Leonard, 1967; Neumann and Prinz, 1974, 1975), deficiency correction capability (Rombola *et al.*, 2000; Fernández *et al.*, 2006, 2008b; Restrepo-Díaz *et al.*, 2008), rate of physiological processes (Swietlik *et al.*, 1982; Orbović *et al.*, 2001; Knoll *et al.*, 2006; Bai *et al.*, 2008), and effect on yield and quality parameters (Brown *et al.*, 1996; Nyomora *et al.*, 2000; Pestana *et al.*, 2001; Wojcik and Szwonek, 2002; Alcaraz-López *et al.*, 2004; Dong *et al.*, 2005; Lester *et al.*, 2006; Singh *et al.*, 2007; Amiri *et al.*, 2008).

Response to foliar nutrient sprays can be remarkable when treatments are applied to deficient plants (Weinbaum, 1988). Indeed, the majority of agronomic foliar nutrient spray studies have been developed with deficient plants and foliar fertilisers have been

traditionally used to correct punctual nutrient deficiencies. However, there is an increasing trend to apply foliar sprays in the absence of deficiency symptoms, at least as it refers to elements with little phloem mobility such as Ca, B, Fe, Mn or Zn. While it is largely recognised that complete reliance on foliar sprays is not commercially viable (Johnson *et al.*, 2001; Fernández *et al.*, 2006), foliar applications prove a valuable means to complementing root treatments and an alternative to supplying nutrients to plants in special situations such as at peak nutrient demand times or in conditions of low soil availability (Weinbaum, 1988; Brown, 1999; Lester *et al.*, 2006).

In general, given the low penetration rates of foliar nutrient solutions, the concentration range employed is higher as compared to root treatments. For instance, trials to assess the effect of micronutrient root treatments are in the μM range versus mM concentrations employed in foliar applications. The need to supply high concentrations to ensure that foliar uptake takes place is more dramatic with macronutrients (e.g, N and K), which are required by plants in greater amounts which are not likely to be met only by foliar treatments (Johnson *et al.*, 2001). Application of concentrations that are too high may unequivocally lead to leaf damage, a common risk associated with foliar sprays. Actually, from the existing foliar fertilisation studies it is not possible to gain information on the optimal element concentration to select for one single element, since the range is amazingly broad (e.g., from 2 to 18 mM Fe) (Fernández *et al.*, 2008b; Fernández and Ebert, 2006) and from 2 M (Castagnoli *et al.*, 1990) to 1 mM Zn (Zhang and Brown, 1999a). In spite of the increased phyto-toxicity risk, there is a common belief that higher leaf-applied solution concentrations will ensure better plant responses. However, several studies have shown a decrease in uptake rates with increasing concentrations expressed as a percentage of the amount applied to the leaf surface (Middleton and Sanderson, 1965; Bowen, 1969; Chamel, 1988; Schlegel *et al.*, 2006). Therefore, development of target studies to elucidate the optimal concentration range to apply according to the particular element-plant species combination will maximise the benefits of foliar fertilisation, minimise costs and reduce environmental impact.

III. STUDYING THE PRINCIPLES OF FOLIAR FERTILIZATION – A HISTORICAL OVERVIEW

Written evidence for the capacity of plant leaves to absorb water from the environment dates back to the 17th century (Mariotte, 1679). However, the absorption of mineral elements by leaves with the subsequent physiological effect in plants was first demonstrated in the 19th century (Gris, 1844; Mayer, 1874; Böhm, 1877). Also at that time Brongniart (1834) described the cuticle as a thin film covering the aerial parts of plants and von Mohl (1847) depicted the layered structure of the cuticular membrane. Various studies regarding gas exchange, transpiration, nutrient assimilation, leaf physiology and anatomy were developed by the end of this century (e.g., Boussingault, 1868; Merget, 1873; Sachs, 1884; Brown and Morris, 1893; Stahl, 1894; van Wisselingh, 1895). The role, number and morphology of stomata received considerable attention as of the beginning of the 20th century (Eckerson, 1908; Muenscher, 1915; Hirano, 1931; Ashby, 1932). Studies to characterise the structure and composition of the cuticle and the epidermis of several plant species were also carried out (Lee and Priestly, 1924; Markley and Sando, 1931; Artz, 1933; Anderson, 1934).

Staining techniques with specific dyes (e.g. fluorescein, Schumacher, 1936; Rhodes, 1937; Palmquist, 1939) were introduced to elucidate the movement of water and solutes in the plant and within the leaf, which were further explored in the following decades (Strugger, 1952; Charles, 1953; Currier and Strugger, 1955; Flasch, 1955).

The penetration and translocation of spray oils into plant leaves and the physical mechanisms involved relating the properties of the solution (e.g., viscosity, surface tension or contact angle), were studied by many authors (e.g., Molisch, 1912; Knight *et al.*, 1929; English, 1930; Hoskins, 1933; Rohrbaugh, 1934; Ebeling, 1939). In parallel, trials to assess the effect of foliar sprays, especially of iron (Fe) were applied to crop plants with variable success (e.g., Wallace 1928; Burke 1932).

The potential contribution of stomata to the foliar uptake process has been controversially discussed since that time (Turrell, 1947). Considering size, shape and surface properties of the stomatal pore of Citrus, the composition of the spray solution and principles of capillarity applied to the penetration of solutions into porous materials as discussed by Hoskins (1933), Ebeling (1939) and later by Adam (1948), it was suggested that a liquid of proper characteristics may penetrate stomata (Turrell, 1947). However, the penetration of citrus leaf stomata by pure water was regarded as unlikely; a problem which has been thought to be solved by applying oil emulsions or wetting agents to the solution (Turrell, 1947). Since then, several investigations assessed the effect of nutrient sprays also in combination with surface active agents such as the highly phyto-toxic, anionic sulfosuccinate ester Vatsol OT (Na-dioctyl-ester of Na-sulfosuccinic acid) (Guest and Chapman, 1949).

To distinguish the routes of entry and movement of foliar-applied aqueous solutions and learn about the relative importance of the stomatal versus the cuticular component of absorption, Dybing and Currier (1959, 1961) and Currier *et al.*, (1964) proposed the use of fluorescent dyes. Working with attached and detached leaves of several plant species, they showed the role of stomatal opening with regard to the process of foliar uptake, in addition to surface-active agents significantly increasing penetration rates. Concerning the effect of stomata on foliar uptake of Fe sprays in combination with Vatsol OT, Wallihan *et al.*,(1964) recorded maximal re-greening rates after treatment when stomata were open.

The use of radioactive isotopes proved useful for investigating the uptake and translocation of elements in the plant (Biddulph, 1940; Overstreet and Broyer, 1940; Broyer, 1950; Chen, 1951). Such techniques were employed to investigate the mechanisms of foliar uptake (Swanson and Whitney, 1953; Gustafson 1956; Bukovac and Wittwer, 1957; Wittwer and Teubner, 1959; Tukey *et al.*, 1961).

Meanwhile, investigations to characterise the chemical composition and structure of the cuticle and the effect of the environment were carried out (Roberts *et al.*, 1948; Linskens, 1950; Skoss, 1955; Orgell, 1955; Martin and Batt, 1958; Hall, 1966; Kolattukudy, 1970), as well as research regarding the basis of leaf wettability and water repellence (Fogg, 1947, 1948; Challen, 1960; Holloway, 1969, 1970). Later these studies were extended to the effects of epiphytic microorganisms (Knoll and Schreiber, 1998, 2000) wax degradation (Cape, 1983; Percy and Baker, 1988) and surface contaminations (Staszewski *et al*., 1994). The principles of self-cleaning properties of leaf surfaces, called “Lotus-Effect” (Barthlott and Neinhuis, 1997) were elucidated (Neinhuis and Barthlott, 1997; Bhushan and Jung, 2006; Guo and Liu, 2007), and recent studies investigated the growth and self assembly of epicuticular waxes by means of atomic force microscopy (Koch *et al.*, 2004; Koch and Ensikat, 2008). Current research focuses on the biosynthesis and transport of cuticular components as well as the underlying genetic control mechanisms (Heredia, 2003; Kunst and Samuels, 2003; Tanaka *et al.* 2004; Nawrath, 2006; Heredia-Guerrero *et al*., 2008).

Many studies showed that the presence of stomata could significantly increase the rate of foliar uptake (e.g., Sargent and Blackman, 1962; Jyung *et al.*, 1965; Kannan, 1969; Norris and Bukovac, 1969; Greene and Bukovac, 1974), chiefly under conditions

favouring stomatal opening (e.g., Wallihan *et al.*, 1964; Middleton and Sanderson, 1965; Sargent and Blackman, 1965; Sands and Bachelard, 1973; Eichert *et al.*, 1998, 2002; Schlegel and Schönherr, 2002; Fernández *et al.*, 2005; Schlegel *et al.*, 2006). The underlying mechanism(s), however, had for a long time been under debate. While some authors assumed a direct penetration of water and solutes through the stomatal pores (Middleton and Sanderson, 1965), others attributed the effects of stomata to a higher permeability of the peristomatal cuticle (Franke, 1967, Sargent and Blackman, 1962; Schönherr and Bukovac, 1978; Schlegel and Schönherr, 2002; Schlegel *et al.*, 2005).

The discussion about the barrier properties of stomata against the penetration of aqueous solutions was preliminarily terminated by Schönherr and Bukovac (1972), who demonstrated that stomata are protected against infiltration of liquids by the combination of three mechanisms: (i) the low wettability of the stomatal pore surface, which is covered by a cuticle, (ii) the high surface tension of water or aqueous solutions, and (iii) the specific geometry of the stomatal pore (Figure 1).

One of the consequences of the prevailing paradigm of a purely cuticular pathway was the reduction of complexity of model systems utilized for the analysis of penetration processes. The vast majority of laboratory studies following the groundbreaking paper by Schönherr and Bukovac (1972) were conducted with astomatous isolated cuticles (e.g. Haas and Schönherr, 1979; Chamel and Gambonnet, 1982; Chamel, 1988; Knoche and Bukovac, 1993, Bukovac and Petracek, 1993; Knoche *et al.*, 2000). The diffusion of lipophilic molecules was also studied in extracted and re-crystallized waxes (Schreiber and Schönherr, 1993; Schreiber *et al.*,1996a; Schreiber and Riederer, 1996). To date substantial progress has been made in the understanding of the properties of the so-called lipophilic pathway (Riederer and Friedmann, 2006).

On the other hand, until the late 1990´s, only a few mechanistic studies dealt with the penetration of hydrophilic solutes through leaf surfaces (Schönherr 1976a; McFarlane and Berry, 1974; Chamel and Gambonnet, 1982; Tyree *et al.,* 1990a,b, 1991, 1992). In retrospect, this research gap is quite astounding, since hydrophilic solutes play an important role in a range of processes, such as the leaching of nutrients out of leaves (Tukey, 1970; Scherbatskoy and Klein, 1983) or the foliar uptake of non-volatile pollutants (Cape, 1993; Sheppard, 1994), some types of crop protection agents (Kirkwood, 1999) and the majority of mineral foliar fertilizers (Tan *et al.*, 1999; Fernández and Ebert, 2006; Fernández *et al.*, 2005).

The publications of Eichert *et al.* (1998) and Schönherr (2000) apparently initiated a renaissance of such research topic which continues to the present day. Meanwhile, a number of studies have been published in which the foliar penetration of polar or ionic species was investigated. Some authors supported the idea that hydrophilic solutes use a cuticular penetration pathway which is physically different from that of the lipophilics (Schönherr, 2000, 2001; Schönherr and Schreiber, 2004; Schreiber, 2005), a hypothesis which was already developed by Schönherr (1976a) who named this pathway “polar pores”. To date only indirect evidence is available for cuticular polar pores, since current progress in microscopical techniques is still unable to detect such nano-structures in biological material (Koch and Ensikat, 2008).

In the past decade, the role of stomata experienced a similar reappraisal by the hypothesis that penetration of solutes may occur not by infiltration but by diffusion in trans-stomatal water clusters (Eichert and Goldbach, 2008; Eichert *et al.*, 2008).

IV. LEAF SURFACE-RELATED FACTORS AFFECTING THE PENETRATION PROCESS

The ultimate parameter controlling the penetration of foliar-applied substances is the intrinsic permeability of the leaf surface, which will be discussed in chapters V. and VI. Other factors modifying penetration rates are related to processes on the leaf surfaces, such as leaf wettability and the dose and concentration of a foliar-applied substance, which determines the driving force of penetration.

The characteristics of the leaf surface and in particular the presence of epicuticular waxes, will determine the rate of retention and wettability of a leaf-applied solution (Koch and Ensikat, 2008). Surface roughness and hydrophobicity will lead to the reduced adhesion of water, contaminants and microorganisms and to the occurrence of large contact angles of water on leaves (Holloway, 1969; Neinhuis *et al.*, 2001; Koch and Ensikat, 2008). Surface wetness is furthermore influenced by atmospheric variables (e.g., irradiation, relative humidity or temperature), physical properties of plants (leaf topography, shape or position in the plant) and initial water distribution (drop volume and film thickness) (Magarey *et al.*, 2005).

While the general surface characteristics of leaves are genetically determined, the actual properties of a given leaf surface depend on other factors, such as the environmental conditions during ontogenesis and the prevailing environmental conditions during foliar treatment, in terms of their effects on the physicochemical behaviour of the applied solutions and the physiological status of the plant. Furthermore, the mode of spray application and the addition of formulation adjuvants will influence foliar penetration. Given the complex scenario surrounding the penetration and physiological effect of foliar sprays only some major factors involved will be detailed below, as follows.

A. Environmental Factors

1. Long Term Effects on Leaf Structure

Foliar spray uptake is determined by many plant-environmental characteristics which will ultimately have a direct influence on factors such as leaf morphology, structure, position, sun exposure or the rate of physiological processes in the plant.

The prevailing environmental conditions during plant growth have a direct influence on the leaf surface in terms of cuticle thickness or amount and composition of epicuticular waxes (Currier and Dybing, 1959; Bird and Gray, 2003; Koch *et al.*, 2006). Some studies showed that light reduction and shading during plant growth induced a decrease in the amount of wax per leaf area (Whitecross and Armstrong, 1972; Baker, 1974). In contrast, high temperatures have been observed to modify the morphology and composition of epicuticular waxes (Whitecross and Armstrong, 1972; Riederer and Schneider, 1990; Welker and Haas, 1999)

Recent evidence suggests that signals from the cuticle may play a role in the environmental control of epidermal cell development, regarding how trichome and stomatal numbers are determined (Bird and Gray, 2003). Actually, the composition of cuticular waxes has been shown to have an influence on the degree of stomatal development in the leaf epidermis. However, it is not clear if cuticular lipids are developmental signalling molecules or whether they are indirectly involved in such developmental signalling pathway (Holroyd *et al.*, 2002; Bird and Gray, 2003). Therefore, environmental conditions can affect the amount and composition of epicuticular waxes, which in turn may influence stomatal and trichome development and ultimately affect the penetration rate of leaf-applied solutes for the same plant species but depend on the particular growing conditions.

Relative humidity has also been shown to modify the amount of wax per leaf area and wax crystal morphology (Baker, 1974; Koch *et al.*, 2006). In a study carried out with *Brassica oleracea*, *Eucalyptus gunnii* and *Tropaeolum majus* by Koch *et al.* (2006), growth at 98 % RH decreased the total amount of wax per leaf area as compared to the effect of lower RH (20-30%), which led to an increase in wax deposition and crystal density on the leaf surface and subsequently, to a reduction in leaf surface wettability. Therefore, it can be concluded that environmental factors may significantly affect the structure of the leaf surface, both at the cuticular and stomatal level and that such variations may alter the response to foliar sprays even for the same plant species grown under different environmental conditions.

Aging of leaves involves passive (e.g., accumulation of injuries, pathogens, biochemical damage and residues) and active processes (i.e, regulated responses to accommodate to the prevailing environment), which may affect the permeability of the leaf surface to water and solutes and water use efficiency (Jordan and Brodribb, 2007; Munné-Bosch, 2007). Leaf surface injuries caused by, for example, abrasion by wind particles or microbial action can have a dramatic effect on the capacity of the leaf to prevent the two-way exchange of water and electrolytes and may become a major pathway for the penetration of foliar sprays.

A reduction in the efficiency of stomatal closure and stomatal control will lead to the deterioration of leaf water status (Jordan and Brodribb, 2007; Munné-Bosch, 2007; Reich and Borchert, 1988). For instance, leaf aging may imply failure to open stomata (e.g. due to re-translocation of K during leaf senescence) (Turner and Begg, 1973), the development of “plugs” as described in Citrus, (Turrell, 1947) or the occlusion of the stomatal antechamber by waxes as observed in conifers and Eucalyptus spp. (Hanover and Reicosky, 1971; Hu and Wang, 1984; Chiu *et al.*, 1992; England and Attiwill,

2006). In general, it is thought that young, partially expanded leaves are more penetrable than fully-expanded leaves (Sargent and Blackman, 1962). However, the significance of leaf aging regarding the penetration of foliar sprays is currently not fully understood and has not been explored in detail. Physical damage of the leaf surface will favour the penetration of foliar sprays, whilst a potential reduction of stomatal opening may decrease the chance of uptake via the stomatal pathway as suggested by Fernández *et al.* (2008a).

Nowadays, there is evidence that signalling is required at several levels to ensure the correct specification, pattern and functioning of stomata (Bergmann, 2006). A recent study showed that the morphological diversity of stomata translates into considerable mechanical and functional diversity regarding leaf gas exchange control (Franks and Farquhar, 2007). However, there is no information regarding the permeability to water and solutes in relation to morphological variations in stomata.

To minimize microbial invasion *via* stomata, it is suggested that stomatal closure is an innate plant defense mechanism for restricting bacterial invasion. Stomatal guard cells of *Arabidopsis* are able to sense bacterial molecules, hence, triggering stomatal closure as a protecting mechanism (Melloto *et al.*, 2006). There is no direct evidence of stomata reacting on the sole contact to solutions. Discussion about stomatal penetration of solutes through stomata has been traditionally restricted to their morphological and physical aspects.

A recent investigation carried out with iron-deficient pear and peach trees grown in calcareous soils showed the impact of this physiological disorder in leaf structure and anatomy (Fernández *et al.*, 2008a). Iron chlorosis was found to decrease the size of stomatal pores in peach and pear leaves and to reduce stomatal conductance. A lower cuticle weight per unit surface was observed in iron-chlorotic pear leaves, while deficient peach leaves had significantly lower amounts of soluble cuticular lipids (Fernández *et al.*, 2008a). Although there is not much information available on the effect of nutrient deficiencies at the leaf structure level, the results reported by Fernández *et al.* (2008a) suggest that they may induce effects at the leaf epidermal level with potential implications for the penetration of foliar sprays.

2. Direct Effects on the Penetration Process

Environmental factors such as relative humidity (RH), temperature (T) and light play an important role in relation to both the behaviour of the spray solution on the leaf surface and the subsequent foliar uptake and distribution process (Ramsey *et al.*, 2005; Currier and Dybing, 1959). Under field conditions, there is a continuous interaction between such factors that will provoke variable responses at a physiological and physical-chemical level, thereby making it difficult to predict the performance of foliar sprays. A brief account of the main effects induced by environmental factors in relation to the potential response to foliar sprays regarding the plant and the physical-chemistry of the solution will be given in the following paragraphs:

Temperature affects chemical reactions and physical properties of plants at the cellular, organ and entire plant level (Gruda, 2005). Currier and Dybing (1959) mentioned the beneficial effects of moderately warm T in stimulating foliar penetration through increasing the rate of physiological processes such as photosynthesis and translocation within the plant. However, high T in combination with low relative humidity as observed over the summer season in most arid and semi-arid areas of the world is likely to limit the rate of spray absorption due to the fast drying of the solution and the lower level of cuticle hydration.

Light is known to stimulate stomatal opening and various physiological processes in the plant such as photosynthesis or xylem flux, which may increase the rate of foliar uptake (Currier and Dybing, 1959; Jyung and Wittwer, 1964). Guard cells may respond directly to environmental cues, such as blue light (Outlaw, 2003), thereby facilitating the involvement of stomata in the foliar penetration process. However, the effects of light on foliar uptake are complex and cannot simply be related to stomatal opening (Middleton and Sanderson, 1965). Several foliar application studies provided evidence for the major role of light in stimulating foliar penetration through the abaxial leaf side (e.g., Wallihan *et al.*, 1964; Greene and Bukovac, 1971; Schlegel *et al.*, 2006) and the process of the exogenous element distribution from the site of application (Fernández *et al.*, 2005).

Stomata control the uptake of CO_2 and the loss of water (Outlaw, 2003). Therefore, stomatal function is critical, and guard cells will respond quickly to physiological and environmental signals. Through the control of transpiration, stomata regulate the leaf's supply of solutes transported in the xylem, such as Ca^{2+}, H^+, K^+ and ABA, all of which are regulators of stomatal aperture and some of which affect guard cell gene expression (Outlaw, 2003). Guard cells also respond to intrinsic foliar signals, such as the concentration of internal CO_2 and apoplastic metabolites of leaves (Outlaw, 2003). Therefore, it can be concluded that stress conditions altering stomatal functioning and plant physiological processes will affect the rate of absorption of leaf-applied chemicals. In this regard, a recent investigation carried out with water and K stressed olive trees, showed an associated decrease in the rate of foliar uptake of leaf-applied K and Rb under plant stress conditions (Restrepo-Díaz *et al.*, 2008).

Factors related to the physiological state of the plant such as root temperature, osmotic potential of the roots, hydraulic conductance or nutrient status will also influence the effectiveness of foliar fertilisation (Weinbaum, 1988; Chamel, 1988).

Relative humidity is a key factor influencing the penetration of foliar-applied solutions, since it will affect both the rate of cuticular hydration and thereby the mechanisms of penetration of solutes through the leaf surface (Fernández *et al.*, 2008a) and the evaporation behaviour of foliar-applied solutions (Eichert *et al.,*1998; Schönherr 2000). These effects are further discussed below in sections IV.B and V, respectively.

B. Physico-chemistry of the Solution

According to Fick's law, the concentration gradient is the driving force for diffusion. The penetration rates of diffusion of any externally-applied solute through the leaf surface thus depend on both its concentration on the leaf surface and its concentration inside the leaf. The concentration of a given solute inside the leaf, i.e., in the epidermal apoplast, depends on the nature of the compound under consideration and on physiological plant factors such as mobility and uptake rate into the epidermal and mesophyll cells (Grignon *et al.*, 1999; Ewert *et al.,* 2000). In comparison to the external concentrations of foliar-applied substances, in particular to the concentrations in the applied droplets or their residues reached after equilibrium with the atmosphere (which can be higher than 10 M, see Section V.C.4.), the internal concentrations underneath such a drop are supposedly much lower, especially at the very beginning of a foliar treatment. It can therefore be concluded that foliar uptake rates are chiefly governed by the external concentration of the solutes.

The relationship between concentration of the applied solution and foliar penetration rates is not completely clear. While a positive relationship between increasing concentrations and penetration rates has been measured in several studies for certain elements and plant species (Schönherr, 2001; Zhang and Brown, 1999a), a different

behaviour has also been reported. Working with Fe-compounds and intact leaves or cuticles, Schönherr *et al.* (2005) and Schlegel *et al.* (2006) measured a negative correlation between increasing concentrations and the penetration rate expressed as a percentage of the applied amount, which, however, represents a high concentration of Fe penetrating the leaf or the cuticle. A similar effect has been described for the penetration of foliar-applied K (Chamel, 1988) and other elements (Tukey *et al.*,1961; Middleton and Sanderson, 1965) and also for 2,4-D (Liu, 2004). Chamel (1988) hypothesised that the decrease in relative penetration rates with higher K concentrations may be due to a progressive saturation of the sites of uptake. An alternative hypothesis to explain the inverse relationship measured with Fe compounds proposes that Fe-salts and chelates may reduce the size of the hydrophilic pathway by inducing the partial dehydration of the pores (Schönherr *et al.*, 2005; Weichert and Knoche, 2006).

In the case of mineral nutrients, initial concentrations of foliar-applied solutions depend on many factors such as plant species, plant age, nutritional status and weather conditions (Wittwer and Teubner, 1959; Wójcik, 2004) and on the type of nutrient (Marschner, 1995). Usually, concentrations of the applied solution are well below 1 M. Once sprayed, the solution starts to evaporate and will, if ambient humidity is too low, eventually dry out whereby the applied compound is immobilized (Allen, 1970). The effect of mobility of a foliar-applied substance and its dependence on ambient humidity has been observed in many studies (Allen, 1960; Middleton and Sanderson, 1965; Forster and Maun, 1980; Grattan *et al.*, 1981, Eichert *et al.*,1998; Schönherr, 2000).

This so far rather qualitative description of processes on leaf surfaces was significantly improved by Burkhardt, who was the first to apply the terminology and quantitative physicochemical treatment of wetting and drying processes of solutes developed in aerosol science in leaf surface-related research (Burkhardt, 1994; Burkhardt and Eiden, 1994). When studying the wetness duration on leaf surfaces he found evidence for leaf wetness at ambient humidities far below saturation and concluded that hygroscopic particles and salts deposited on the leaf surfaces dissolve in water absorbed from the surrounding atmosphere, if the relative air humidity (RH) is above the deliquescence relative humidity (DRH) of the respective salt (Burkhardt, 1994). Whereas the term deliquescence describes the dissolution of a salt occurring whenever ambient RH reaches or exceeds DRH, the reversed process, the drying of a solution and crystallization of the solute when RH falls below a certain RH, is called efflorescence. The RH, at which drying occurs, is called efflorescence relative humidity (ERH), which thermodynamically equals DRH. Often, there is a substantial hysteresis, i.e. solutions can be supersaturated and crystallize at RH values below the DRH (Pilinis *et al.*, 1989).

When humidity rises from dry (RH = 0%) to saturation (RH = 100%), dry salt crystals suddenly dissolve in absorbed water as soon as RH reaches DRH. The salt concentration at this point is at its maximum, and thus can be expressed by its water solubility, which is a physical constant at a given temperature. If RH >DRH of the compound under consideration, it is present in a dissolved state and can thus penetrate the leaf surface, whereas at RH < DRH, the compound is solid and immobile. This dependence of the mobility of a foliar-applied substance on ambient RH has been utilized to predict penetration processes through leaf surfaces and isolated cuticles (Eichert *et al.*, 1998; Schönherr 2000, 2001; Schönherr and Luber, 2001).

C. Droplet Size and Spray Application

Most studies related to spray application techniques and their performance have been carried out with pesticides and herbicides (Currier and Dybing, 1959; Foy, 1964; Knoche, 1994; Leaper and Holloway, 2000; Farooq *et al.*, 2001; Steiner *et al.*, 2006; Sanyal *et al.*, 2006; Wang and Liu, 2007).

Appropriate spray application is a key process ruling the effectiveness of a leaf-applied solution. The spray-application process involves a series of complex inter-related events, namely, formulation of an active ingredient, atomisation of the spray solution, transport of the spray to the target plant surface and droplet impaction, spreading and retention on the leaf surface, residue formation and penetration into the leaf (Brazee *et al.*, 2004).

Application of a foliar treatment implies that the liquid is passed through a spray generating system to produce droplets, which are commonly pressure nozzles (Butler Ellis *et al.*, 1997). This method of treatment is inherently inefficient, since only a limited amount of liquid meets the plant given the occurrence of losses relating to, for example, droplet reflection, run-off, drift and in-flight evaporation (Shaw *et al.*, 1987; Leaper and Holloway, 2000; Wang and Liu, 2007). The principal factors that are influenced by nozzle performance are the risk of spray drift, the distribution of the deposit on target surfaces and the mode of action of the active ingredient on the leaf surface. On the other hand, nozzle performance depends on the interaction between the physical-chemical properties of the spray solution and nozzle design (Miller and Butler Ellis, 2000). Spraying efficiency can also be improved by changing spray volume, either with an increase in pressure or a change in nozzle tip or atomizer type (Shaw *et al.*, 1987). Apart from the characteristics of the nozzle and the solution, the trapping efficiency of the plant canopy depends on many features including the prevailing climate, and the orientation, shape and size of the biological target (Shaw *et al.*, 1987; Bukovac *et al.*, 2002). If leaves are wet through rain or dew or a large volume of spray is applied, there is an increased potential for reflection or run-off and retention will decrease (Zabkiewicz, 2002). Droplet size and velocity distributions determine the delivery and retention of the spray on the target plants (Butler Ellis *et al.*, 1997). Spray efficacy often depends on droplet size (Hislop, 1987), better coverage being achieved with smaller droplets which are more likely to be retained on the leaf surface but are more prone to drift (Butler Ellis *et al.*, 1997; Tuck *et al.*, 1997).

Development of models to predict droplet size and spray performance under field conditions proves difficult due to the many factors involved and the more complex nature of spray mixtures (Miller and Butler Ellis, 2000; Liu, 2004; Steiner *et al.*, 2006).

Changing the properties of the spray solution by addition of adjuvants will influence the formulation process and also the mechanisms of spray formation and drop performance on the leaf surface (Miller and Butler Ellis, 2000). Many types of adjuvants are being used in foliar spray formulations, the most widely used being surfactants (Wang and Liu, 2007). The addition of adjuvants can induce significant changes in the quality of the spray, with effects on droplet size, velocity and structure (Butler Ellis *et al.*, 1997). Spread areas of droplets can be modified by the addition of surface-active agents, extensive spreading leading to coalescence of spray droplets with the subsequent decrease in retention and increased run-off from the leaf surface (Knoche, 1994).

D. Formulation Adjuvants

The presence and topography of epicuticular waxes will render the leaf surface difficult to wet (Schönherr, 2000) and depending on their characteristics (Neinhuis and

Barthlott, 1997) it is possible that the drops of an aqueous solution may only make contact with the tips of wax crystalloids. The addition of adjuvants and, in particular, surface-active agents to modify the physical-chemical properties of the spray solution, can enable effective wetting of the leaf surface (Schönherr, 2000).

Adjuvants are defined as any substance in a formulation or added to the spray tank that modifies active ingredient activity or spray characteristics (Hazen, 2000). Adjuvants are generally classified as: (i) activator adjuvants (e.g., surfactants) which increase the activity, penetration, spreading and retention of the active ingredient and, (ii) utility adjuvants (e.g., buffering or compatibility agents) that modify the properties of the solution without directly affecting the efficacy of the formulation (McMullan, 2000; Penner, 2000). The potential of activator adjuvants and, in particular, surface-active agents, to improve the efficacy of foliar sprays has long being recognised (Moore and Graham, 1918; Guest and Chapman, 1949; Jansen *et al.*, 1961; Ramsey *et al.*, 2005). Since the efficiency of an agrochemical active ingredient is often limited if applied to the leaf surface alone (Perkins *et al.*, 2005), adjuvants that accelerate and promote the process of foliar penetration via, for example, enhancing leaf wetting, retention, penetration and sometimes humectancy are commonly included in spray formulations (Tamura *et al.*, 2001; Liu, 2004; Schönherr *et al.*, 2005).

Addition of humectants such as hygroscopic compounds to prolong the process of spray drying may be a key factor in improving the performance of nutrient sprays, especially in arid and semi-arid areas of the world (Currier and Dybing, 1959; Schönherr *et al.*, 2005; Fernández *et al.*, 2006). Several investigations showed that the addition of formulation adjuvants such as surfactants (Weinbaum and Neumann, 1977; Chamel, 1988; Weinbaum, 1988; Wójcik, 2004; Schlegel *et al.*, 2006; Fernández *et al.*, 2008b) and additives like urea, dimethylsulfoxide (DMSO) or glycine-betaine (Leonard, 1967; Chamel and Simiand, 1970; Basiouny and Biggs, 1971; Chamel, 1988; Weinbaum, 1988; Díaz-Zorita *et al.*, 2001; Fernández *et al.*, 2006) can significantly improve the performance of foliar sprays.

Surfactants are actually the most widely-used and efficient additives in enhancing foliar spray efficacy (Wang and Liu, 2007). However, the implicit phyto-toxicity of most surface-active agents was reported since surfactants were included in agrochemical formulations (Jansen *et al.*, 1961). Nowadays, there is agreement that leaf uptake activation is the end product of the interactions between the surfactant, the agrochemical and the leaf surface, processes which are not directly related to the actual surface-active properties of the surfactant itself. However, surfactant composition and concentration will be key factors influencing the performance of the active ingredient (Stock and Holloway, 1993). The actual mode of action of surfactants on absorption of the active ingredient is not fully understood (Hess and Foy, 2000; Knoche and Bukovac, 1992; Ramsey *et al.*, 2005; Wang and Liu, 2007)

Surfactants are by necessity large molecules, consisting of a non-polar, hydrophobic portion, attached to a polar or ionic, hydrophilic group (Cross, 1998; Tadros, 1995; Hess and Foy, 2000). It is important that the ends of the hydrophobic and the hydrophilic part of the surfactant molecule are sufficiently separated from each other, so that they react independently with surfaces and solvent molecules (Cross, 1998). The hydrophilic portion can be non-ionic, ionic or zwitterionic, accompanied by counter-ions in the last two cases (Tadros, 1995). The occurrence of interactions between electrolyte solutions and surfactants will be theoretically minimised with non-ionic surfactants, which are widely used in agrochemical formulations. The most common non-ionic surfactants are based on ethylene oxide (EO) groups as a hydrophilic component (Hess and Foy, 2000; Ramsey *et al.*, 2005). Stock and Holloway (1993) suggested that uptake of lipophilic

pesticides is generally favoured by the addition of nonionic surfactants with low EO content, which are good spreaders and have low surface tension, whereas uptake of water-soluble pesticides is best with surfactants of higher EO content with poor spreading properties. However, conflicting evidence regarding the effect and possible mechanism of action of high and low EO-content surfactants suggests that ethoxylated surfactants may enhance the uptake of hydrophilic and lipophilic compounds by different means, which are not fully understood so far (Kirkwood, 1993; Gaskin and Holloway, 1992; Haefs *et al.*, 2002; Ramsey *et al.*, 2005).

Regarding the possible mechanisms of surfactant action, Stock and Holloway (1993) suggested that they may: (1) increase the effective contact area of deposits, (2) dissolve or disrupt epicuticular waxes, (3) solubilise agrochemicals in deposits, (4) prevent or delay crystal formation in deposits, (5) retain moisture in deposits and, (6) promote stomatal infiltration. However, it is now recognised that surfactants can also affect the transcuticular diffusion of substances via cuticular solubilisation or hydration and that they can affect the permeability of the plasma membrane. Wang and Liu (2007) concluded that the mechanisms of action of surfactants are very complex and only partially understood.

Low EO-content surfactants that enhance uptake of lipophilics do so by altering the physical properties of cuticles (Coret and Chamel, 1994, 1995; Ramsey *et al.*, 2005). Coret and Chamel (1993, 1995) showed that surfactants with high EO-contents increased hydration of the cuticle.

Following the mechanisms of action described above, surfactants may alter the structure of epicuticular waxes, penetrate into the cuticle and modify its permeability characteristics (Tamura *et al.*, 2001). Several studies showed that surfactants may alter the structure of the leaf surface (Noga *et al.*, 1987; Kuzych and Meggitt, 1983; Feng *et al.*, 1999; Tamura *et al.*, 2001; Perkins *et al.*, 2005). Surfactant-induced ethylene production has been also shown for several plant species and may be related to phyto-toxicity (Lownds and Bukovac, 1989).

Organosilicons are a group of non-ionic surfactants with alkylsiloxane groups forming the hydrophobic moiety (Knoche, 1994). Such surfactants have been found to promote stomatal infiltration by significantly lowering surface tension (Knoche, 1994; Schönherr *et al.*, 2005). They also very much improve leaf wetting and spreading, while retention rates are low (Stevens, 1993; Knoche, 1994). Many reports investigated the effect of organosilicon surfactants on foliar uptake of agrochemicals, and they were also found to have an associated high phyto-toxicity risk (Neumann and Prinz, 1974, 1975; Horesh and Levy, 1981; Stevens, 1993; Knoche, 1994; Fernández *et al.*, 2008b).

In contrast, Uhlig and Wissemeier (2000) showed a reduction of non-ionic surfactant (Triton X-100 and Genapol) phyto-toxicity by the addition of divalent cations (chiefly Ca and to a lower extent, Mg). A relation between increased phyto-toxicity of non-ionic surfactants with decreased lengths of the hydrophilic group has been observed. Surfactant molecules with either large hydrophobic groups, long hydrophilic chains or both were found to be less toxic because of their lower water solubility and, hence, slower penetration (Parr, 1982).

In conclusion, there is evidence that interactions between foliar spray additives occur, but nowadays it is not yet possible to predict the performance of a certain active ingredient in combination with a particular surfactant (Liu, 2004; Ramsey *et al.*, 2005; Wang and Liu, 2007; Fernández *et al.*, 2008b).

V. PERMEABILITY OF ASTOMATOUS CUTICLES TO HYDROPHILIC SOLUTES

A. Structure and Physico-chemistry of the Cuticle with regard to Permeability

The cuticle is a continuous bio-polymer layer synthesized by the epidermis of fruits, leaves, primary stems and organs of flowers (Pollard *et al.*, 2008). The structure and composition of the cuticle and cuticular waxes vary greatly between plant species and varieties, organs and according to different developmental stages (Leide *et al.*, 2007; Heredia-Guerrero *et al.*, 2008).

The cuticle is basically composed of a polymer cutin and/or cutan matrix with waxes both embedded in (intracuticular) and deposited on the surface (Heredia-Guerrero *et al.*, 2008; Pollard *et al.*, 2008). Cuticular waxes are a mixture of hydrophobic compounds chiefly composed of aliphatic lipids (e.g., long-chain fatty acids and their derivatives), and other compounds, such as triterpenoids and phenylpropanoids (Jetter *et al.*, 2006; Nawrath, 2006). Variable amounts of polysaccharide fibrils and pectin lamellae may extend from the cell wall, binding the cuticle to the underlying tissue (Bargel *et al.*, 2006; Jeffree, 2006). Intracuticular waxes are chiefly responsible for the barrier properties to the exchange of water and solutes between the plant and the surrounding environment (Riederer and Schreiber, 1995; Vogg *et al.*, 2004; Richardson *et al.*, 2007), while epicuticular waxes influence the wettability (Holloway, 1969), light reflection (Pfündel *et al.*, 2006) and the self-cleaning properties of the leaf surface (Bargel *et al.*, 2006). Variation in cuticular structure and composition will result in differences in leaf wettability, retention and penetration of aqueous solutions (Riederer, 2006). It was suggested that the cuticle above the veins and in the vicinity of stomata is more penetrable than in other areas of the leaf (Maier-Maercker, 1979a; Schlegel and Schönherr, 2002; Schlegel *et al.*, 2006; Schönherr, 2006).

According to the predominant model, the cuticle consists of two distinguishable layers, the cuticle proper (CP) as the outermost portion, and the subjacent cuticular layer (CL). While the CL contains substantial amounts of cell wall polysaccharides, the CP is by definition free of polysaccharides (Jeffree, 2006), as confirmed by a number of microscopic studies (Norris and Bukovac, 1968; Sargent, 1976; Singh and Hemmes, 1978). By contrast, Hoch (1979) observed that polysaccharide microfibrills in apple cuticles “occasionally” reached the surface of the cuticles. Jeffree (1996) pointed out that the apparent lack of a cuticle proper might be caused by an unperceived disruption of this layer during sample preparation, which was also reported by Wattendorff and Holloway (1984).

Due to its composition and structure, the cuticle is regarded as a lipophilic membrane (Nawrath, 2006). Accordingly, the vast majority of mechanistic studies on cuticular penetrability have been conducted with lipophilic solutes. Meanwhile, the solution-diffusion model is widely accepted to describe the penetration of lipophilics across cuticles (Riederer and Friedmann, 2006). According to this model, penetration rates of a molecule are proportional to its solubility and its mobility in the cuticle (Schreiber, 2006). Based on these two parameters, the permeability can be expressed as:

$$P = D * K / \Delta x \quad (1)$$

where P is the permeance (m s^{-1}), D (m^2 s^{-1}) is the diffusion coefficient of the molecule in the cuticle, which is a measure of mobility, K is the partition coefficient of the molecule between the external solution and the cuticle as a measurement of

solubility, and Δx (m) is the path length of diffusion. The path length is usually much larger than the thickness of the cuticle, and it could be the case that this tortuosity is determined by the waxes embedded in the polymer matrix (Schönherr and Baur 1994, Baur *et al.*, 1996; Baur *et al.*, 1999a) and the spatial disposition of cutin and/or cutan molecules.

On a molecular level, both the solution and diffusion of a molecule in the cuticle can be viewed as jumping into and between voids in the polymer matrix arising by molecular motion (Schönherr, 2006). This view is supported both by studies with synthetic polymers (Crank and Park, 1968) as well as by the observation that penetration of lipophilics through cuticles is strongly enhanced by increasing temperatures. Increasing temperatures may increase the size and/or frequency of voids in the cuticle, which become large enough to accommodate the permeating molecule (Schönherr, 2006). Synthetic plasticisers are thought to increase the fluidity of the cutin network (Schönherr, 1993; Schönherr and Baur, 1994), thereby promoting the penetration of solutes through the cuticle.

The solubility of a compound in the cuticle is usually expressed as the cuticle/water partition coefficient (K_{CW}), which is frequently approximated by its octanol/water partition coefficient, K_{OW} (Kerler and Schönherr 1988; Schreiber *et al.,* 1996b). The latter coefficient is more convenient to use because K_{OW} values of many compounds have already been determined or can easily be measured experimentally. Whilst the K_{OW} and the K_{CW} of lipophilics were reported to be highly correlated (Kerler and Schönherr 1988, Popp *et al.,* 2005), this seems not to apply to polar molecules (Popp *et al.,* 2005).

Since diffusion coefficients (D) in the cuticle are usually not known and can not be directly determined, permeability was often related to molecular mass (Schönherr and Schreiber, 2004; Schlegel *et al.*, 2005) or volume (Bauer and Schönherr, 1992; Buchholz *et al.,* 1998; Popp *et al.,* 2005). In most cases, a close negative linear relationship was found between the logarithm of permeability and molecular size (e.g., Buchholz *et al.*, 1998; Popp *et al.*, 2005). Plots of log (permeability) vs. size were utilized to further characterize the lipophilic pathway. The slope of such a graph was interpreted as the size selectivity of the cuticle, indicating how permeability decreases when the size of the molecules increases. Size selectivities of cuticles of a variety of species were reported to be very similar (Buchholz *et al.*, 1998) and not affected by extraction of waxes (Baur *et al.*, 1996, 1999a). However, the intercepts with the y-axis, i.e. the theoretical permeabilities of a molecule with zero volume or mass, differed substantially between different species (Buchholz *et al.*, 1998) and were strongly increased by wax extraction (Baur *et al.*, 1999a). This was taken as evidence that lipophilics diffuse in the cutin matrix and in the amorphous fraction of waxes, both having a similar chemical and structural constitution in cuticles of different species, whereas the properties of the main diffusion barrier, the crystalline waxes, can differ substantially between species (Buchholz *et al.*, 1998; Baur *et al.*, 1999a).

B. Pathways for the Diffusion of Hydrophilic Solutes – the Hypothesis of "Polar Pores"

Penetration of polar solutes (Stock *et al.*, 1992; Stammitti et al,. 1995; Popp et al,. 2005; Eichert and Goldbach, 2008) and ions (Yamada *et al.*, 1964; Kannan, 1969; Schönherr, 2000; Schönherr and Schreiber, 2004) through intact astomatous cuticles has been unequivocally demonstrated by direct means. Likewise, measurement of electrical conductivity and potentials through plant cuticles provided indirect evidence for the

penetration of ions (Tyree *et al.*, 1990a,b, 1991, 1992; Heredia and Benavente, 1991; Ramos-Barrado *et al.*, 1993).

However, virtually all experiments in which the applied solutions were free from surface-active additives indicated a much lower permeability of cuticles for ions than for lipophilic compounds. In the study of Yamada *et al.* (1964), for instance, the average hourly penetration rate of ions through tomato fruit cuticles was only 0.005% to 0.05% of the applied dose. Studying the penetration of $CaCl_2$ across pear cuticles Schönherr (2000) reported values of 0.2% per hour. It was also stated that organic acids penetrate cuticles more slowly in the ionised than in the non-dissociated form (Niederl *et al.*, 1998) and that ionised organic acids cannot penetrate cuticles at all (Schönherr *et al.*, 2000). Much lower penetration rates of hydrophilic than of lipophilic solutes are in agreement with their low solubility in the lipophilic cuticle, which according to the solution-diffusion model results in low permeances (Equation 1).

Some authors, however, concluded that the penetration rates of hydrophilics are still too high to be explained by simple solution and diffusion in the cuticle and proposed that hydrophilic solutes penetrate cuticles via a physically distinct pathway, which they entitled "polar pores" (Schönherr, 1976a) or "aqueous pores" (Schönherr, 2000; Schönherr and Schreiber, 2004) or "water-filled pores" (Schreiber, 2005). It was suggested that these pores are generated by the adsorption of water molecules to polar moieties located in the cuticular membrane (Schönherr, 2000; Schreiber, 2005), such as unesterified carboxyl groups (Schönherr and Bukovac, 1973), ester and hydroxylic groups (Chamel *et al.*, 1991) in the cutin network and carboxylic groups of pectic cell wall material (Schönherr and Huber, 1977). This hypothesis is supported by studies on cuticular water sorption indicating that adsorbed water molecules can form clusters in the cuticle (Luque *et al.*, 1995b). On the other hand, some authors did not find experimental evidence for the presence of pores in leaf cuticles (Kerstiens, 1996a; Niederl *et al.,* 1998).

Since the estimated size of cuticular pores is below the accessible microscopical resolution in plant cuticles, the existence of the putative pores has not yet been visualized (Koch and Ensikat, 2008). Thus this hypothesis still rests on indirect evidence. Schreiber (2005) stated five arguments pointing to the existence of such polar pores, namely: (i) Penetration of ions was reported to be independent of temperature and (ii) of plasticizers, and (iii) only weakly affected by wax extraction, (iv) is affected by humidity and (v) was reported to be less size-selective as compared to the lipophilic path. Additional arguments for the existence of two distinct cuticular pathways come from studies on the size exclusion of large hydrophilic molecules, which were interpreted as evidence for a finite size of the polar pores (Schönherr 1976a; Popp *et al.*, 2005), and electrochemical studies indicating a different penetration behaviour of cations and anions (e.g. Tyree, 1990a; Benavente *et al.*, 1999).

1. Effects of Temperature, Plasticizers and Wax Extraction

To date there are only a few studies in which the effect of temperature on the penetration of polar compounds was analysed. Temperature effects are usually expressed as activation energies of diffusion, which are represented by the slopes of Arrhenius graphs, i.e. plots of mobility of a compound against 1/T. Activation energies of diffusion are interpreted as the energy required to produce free volumes in a polymer large enough to accommodate the diffusing molecule (Baur *et al.*, 1997). Consequently, activation energies increase with an increase in the size of the molecule and a rise in temperature increases penetration rates. Typical values of activation energies for lipophilic substances range from 75 to 190 kJ mol^{-1} (Baur *et al.*, 1997). However, for

ions, either no temperature effect was detected or activation energies were much lower than for lipophilic molecules. Schönherr (2000) studied Ca^{2+} penetration through pear leaf cuticles in the range of 15 to 30 °C and could not observe a significant temperature effect. Likewise, Schönherr and Luber (2001) did not find a significant effect of temperature in the range of 10 to 25 °C on the penetration of Rb^{+} through pear leaf cuticles, neither did Popp *et al.* (2005) regarding xylose penetration through ivy leaf cuticles in the range of 15–35°C. Schönherr (2002, 2006) reported activation energies of 2.9 to 68 kJ mol^{-1} for proline and glyphosate salts measured at temperatures between 10 to 25 °C.

At first sight, the striking difference between activation energies of lipophilic and hydrophilic solutes strongly supports the hypothesis of the occurrence of two physically separated uptake routes with totally different physicochemical properties. The high activation energies measured with lipophilic compounds, which increase with an increase of the size of the permeating molecule, support the view that diffusion takes place in the cutin network, and that an increase in temperature increases the probability of formation of free volumes, large enough to accommodate the diffusing molecule. The much lower (or lack of) temperature effect on ion penetration could be interpreted as evidence that diffusion occurs in a pathway where interactions with the diffusion medium are less pronounced, such as in "polar pores". However, it must also be taken into account that ions are on average much smaller than the more lipohilic, organic compounds, which implies that they require per se much smaller void spaces inside the cuticular membranes than the large molecules. Hence, lower activation energies measured with ions could therefore be the consequence of their smaller size rather than of their charge.

Schönherr (2000) did not find a significant effect of the plasticisers tributyl phosphate and diethyl suberate, on Ca^{2+} penetration through pear cuticles. By contrast, Schönherr (2002) reported a comparatively low (rate constants increased by 35%), but significant effect of diethyl suberate on glyphosate anion penetration through *Populus canescens* cuticles. Shi *et al.* (2005) showed that ethoxylated alcohols significantly increased the penetration of the polar methylglucose through isolated *Stephanotis floribunda* and pear cuticles (rate constants increased up to 4), the accelerator effect increasing with a higher degree of ethoxylation.

Schönherr (2000) reported that wax extraction increased Ca^{2+} penetration through pear cuticles by a factor of 1.8 to 2.9, and concluded that wax removal affected Ca^{2+} penetration rates much less than the penetration of lipophilic compounds. This is in contrast to the results of Popp *et al.* (2005) who found no clear difference in the effect on wax extraction between hydrophilic polar compounds (factors ranging from 1.9 to 993) and lipohilic compounds (222-418). On the contrary, size selectivity of penetration of polar compounds was much more affected by wax extraction than that of lipohilics.

2. Effect of Relative Humidity

In a range of studies it was shown that the penetration rates of ions increase with increasing RH. An increase of RH from 50% to 90% doubled the rate constants of penetration of $CaCl_2$ (Schönherr, 2000). In the study by Schönherr (2002), the rate constants of penetration of IPA-glyphosate increased by a factor of 7 when humidity was increased from 70% to 100%. Similar RH effects were shown for $Ca(NO_3)_2$ and organic Ca-salts (Schönherr, 2001) and Rb-salts (Schönherr and Luber, 2001). It was suggested that high humidity enhances the transport capacity of the penetration route of hydrophilic solutes by increasing the size or number or polar pores (Schönherr, 2001).

3. Size Selectivity and Pore Size Estimations

Schönherr and Schreiber (2004) studied the size selectivity of diffusion of Ca-salts through astomatous *Populus canescens* cuticles and concluded that it is by far less pronounced than that of lipophilic compounds in *Populus alba* cuticles. On the contrary, in the study of Popp *et al.* (2005), which is the only one in which both lipophilic and hydrophilic molecules were contrasted with the same species and under identical conditions, the hydrophilic path was more size selective than the lipophilic path.

The first attempt to analyse the pore size, i.e. the cross sectional area of an individual aqueous pore, was carried out by Schönherr (1976a) who analysed water fluxes through isolated, dewaxed *Citrus aurantium* cuticles. His calculations yielded radii of about 0.5 nm. However, it must be emphasised that his approach failed to detect pores in intact (i.e., not dewaxed) isolated cuticles (Schönherr, 1976b).

For a long time his values have been the only available estimations of pore sizes in cuticles, and have therefore been frequently cited in the literature and in textbooks (e.g. Marschner, 1995). During this time, the value of 0.5 nm virtually took a life of its own, because it was more and more overlooked that, strictly speaking, it is valid only for isolated and dewaxed astomatous *C. aurantium* cuticles, and that the properties of intact cuticles and /or of other plant species may be significantly different. Later, Popp *et al.* (2005) estimated pore radii of the hydrophilic route in isolated *Hedera helix* cuticles from the size selectivity of diffusion of polar organic molecules and reported pore radii of 0.5 nm and 0.3 nm. Eichert and Goldbach (2008) following a co-application approach measuring the penetration of two compounds simultaneously, estimated pore radii of the hydrophilic path for various plant species (*Vicia faba, Coffea arabica, Prunus cerasus, Prunus laurocerasus, Populus x canadensis*). According to their calculations, pore radii in astomatous leaf surfaces ranged from 2.0 to 2.4 nm, values considerably larger than the ones estimated previously.

4. Electrochemical Penetration Studies

Penetration of non-ionic solutes is driven by a chemical potential gradient due to concentration differences between both sides of the leaf surface. However, penetration of ions involves a second gradient, which is the electrical potential gradient caused by the charges of the permeating ions. Whenever anions and cations penetrate in different rates, charge balance is disturbed causing an electrical potential gradient, which under certain circumstances can be the dominant driving force and can even induce the movement of an ion against a concentration gradient (Tyree *et al.*, 1990b). Native plant cuticles carry fixed negative net charges due to the presence of carboxylic groups (Schönherr and Bukovac, 1973; Schönherr and Huber, 1977). It was shown that the negative net charge can, under certain circumstances which are further discussed below, account for a preferential penetration of cations over anions due to anion repulsion by the negatively charged cuticle (Tyree *et al.*, 1990a,b, 1991, 1992). Cations penetrating faster than anions will induce an electric potential difference, which increases with a preceding preferential cation penetration, but which will cease at a certain point when electric potential difference counterbalances the opposite directed chemical potential difference (Riederer, 1989). The charge balance between cation and anions can be restored either by the symport of anions, i.e., cations diffuse together with anions, or by the antiport of cations, i.e., the penetrating cation displaces other cations, probably mainly Ca^{2+} and Na^{+}, which are preferentially sorbed by the negative charges of the cuticle (Heredia and Benavente, 1991), or a combination of both processes. In the case of a symport of cations and anions, penetration rates of the cation are governed by the slower, rate limiting penetration rate of the anion, whereas the antiport of exchangeable

cations is independent of the anion. Scherbatskoy and Tyree (1990) concluded that the latter is the dominating process in foliar leaching of cations. On the other hand, foliar uptake of anions such as nitrate (Peuke *et al.*, 1998; Tan *et al.*, 1999) or phosphate (Clarkson and Scattergood, 1982; Barel and Black, 1979) directly indicates that symport of cations and anions does occur.

The transport properties of mainly fruit cuticles (tomato and pepper) were extensively studied using electro-kinetic measurements (Heredia and Benavente, 1991; Benavente and Heredia, 1992; Benavente *et al.*, 1993, 1997, 1998, 1999; Ramos-Barrado *et al.*, 1993). Comprehensive studies by Tyree and co-workers (Tyree *et al.*, 1990a,b, 1991, 1992) particularly addressed the question of a preferential cation penetration through the leaf cuticle. They measured electrical potentials through isolated cuticles of *Acer saccharum* and/or *Citrus aurantium*, which developed when both sides of the cuticles were exposed to varying concentrations of a range of different chloride salts. Based on the measurements, they calculated permeabilites of individual monovalent and bivalent cations and chloride through the cuticles and developed models on the mechanisms of ion penetration. Their initial hypothesis, the "charged pore" model, can be summarized as follows (Tyree *et al.*, 1990a): Ions move in polar pores, which are associated with the polar components of the cuticle bearing negative charges. Charge density is highest at the inner side of the cuticle and decreases to the outside because fewer carboxylic groups are present. The fixed negative charges in the pores induce a double layer, from which anions are excluded (Donnan exclusion). The thickness of this anion excluding double layer increases with increasing fixed-charge density and decreasing ionic strength. As soon as the double layer lining the pore surface is thick enough to occlude the entire pore, anions are excluded from penetration. Assuming a constant pore diameter, anion exclusion is most likely to occur at the inner layers of the cuticle, because here the asymmetric structure of the cuticle causes the highest charge density. In the subsequent bilayer-model (Tyree *et al.*, 1991), the structural heterogeneity of the cuticle was taken into account. In this model approach, it was assumed that the permeability of the cuticle is rate-limited by the outermost cuticle proper, which is virtually free of carbohydrates and thus bears only a few fixed negative charges.

According to these studies, anion exclusion is most likely to occur when the solute concentration (ionic strength) is low. Under such conditions, only cations can penetrate the cuticle, and only ion exchange of cations absorbed in the cuticle against the penetrating cations can account for appreciable penetration rates (Scherbatskoy and Tyree, 1990). If foliar-applied salts are highly concentrated or as soon as a significant amount of water has evaporated from lowly concentrated foliar-applied solutions, solute concentrations (ionic strengths) could be high enough to suppress anion exclusion. Electro-kinetic studies indicate that this could occur at concentrations > 10^{-2} M (Benavente *et al.*, 1999). In the absence of Donnan exclusion, the permeability of the cuticle is determined by the mobility of the ion in the cuticle, i.e., chiefly by its size (McFarlane and Berry, 1974; Benavente *et al.*, 1999). This might explain why, in contrast to the above-mentioned electrokinetic laboratory studies, there were usually no significant differences in foliar uptake rates of anions and cations reported in many field and laboratory studies (e.g., Bowman and Paul, 1992; Tan *et al.*, 1999; Eichert and Goldbach, 2008).

C. Critical evaluation of the current status of research

1. Significance of Isolated Astomatous Cuticles as Model Systems

The majority of mechanistic studies on the barrier properties of leaf surfaces were conducted using isolated astomatous cuticles. The use of stomata-free cuticles as a simplified model system offers a variety of advantages. First, the mechanisms of penetration can be studied while the controlling factors such as concentration gradients and humidity can be controlled. Second, such a model system enables the direct and unequivocal quantification of penetration rates by measuring the amount of a test molecule recovered in a receiver solution after applying it to the opposite side of the cuticle. In contrast, experiments using intact leaves require reliable washing procedures to remove residues on the leaf surface and to ensure that only substances that actually penetrated the leaf surface are determined (Wallihan and Heymann-Hirschberg, 1956; Smith and Storey, 1976; Orphanos, 1977; Reed, 1983; Baker *et al.*, 1983, Stevens *et al.*, 1988).

However, apart from the fact that the impact of stomata is disregarded, using astomatous isolated cuticles for mechanistic studies on foliar penetration processes requires that the isolation process does not alter the barrier properties of the cuticle. The most common method for isolation of cuticles is based on the enzymatic separation of the cuticle from the underlying cell wall of the epidermis using cellulase and pectinase at low pH (Orgell, 1955; Schönherr and Riederer, 1986; Chaumat and Chamel, 1990). Thereafter the cuticles are sometimes subjected to an alkaline washing step (Schönherr and Riederer, 1986) and flattened under a stream of pressurized air (Kirsch *et al.,* 1997). Other procedures are based on chemical isolation using concentrated hydrochloric acid and $ZnCl_2$ (Holloway and Baker, 1968; McFarlane and Berry, 1974) or oxalic acid (Wattendorff and Holloway, 1982). The final separation step involves washing away cell debris with a jet of water, occasionally followed by further mechanical cleaning, e.g. with a rubber scraper (Ramirez *et al.*, 1992) or a brush (Stammitti *et al.*, 1995).

Such a harsh procedure (Kirsch *et al.*, 1997) could induce the occurrence of artefacts such as leaks or a less evident alteration of the chemical and structural composition of the cuticle. Tyree *et al.* (1990a) assumed that the isolation procedure accounted for the large variability of their results, and wondered whether the enzymatic isolation procedure also digested parts of the intra-cuticular cell wall material.

To detect the presence of holes and therefore discard defective, enzymatically isolated cuticles, Darlington and Cirulis (1963) used the fluorescent dye fluorescein as a marker and observed that 65-90% of the freshly isolated cuticles were leaky. Later, Schönherr (2000) and Schönherr and Schreiber (2004) performed leak tests with pure ethanol before studying their permeability. They found that up to 70% of the cuticles had to be discarded. The very high percentage of cuticle damage following isolation casts serious doubts on the significance of studies with isolated cuticles, in which research was done without assessing the integrity of the cuticular membranes. This problem urgently needs further investigation. On the other hand, it was also never tested whether the pre-treatment of cuticles with ethanol affected the barrier properties of the cuticles in subsequent penetration experiments.

Besides, the permeability of cuticles could also be altered in a more subtle manner by modification of their molecular composition or structure. It is astonishing that only a few investigations tried to address this problem, although the majority of studies on barrier properties were conducted with isolated cuticles. Kirsch *et al.* (1997) concluded that the enzymatic isolation procedure did not affect the permeability of cuticles for

lipophilic organic compounds. In other studies the structure and morphology of the leaf cuticle was found not to be altered by isolation of the cuticle (Wattendorff and Holloway, 1982; Gouret *et al.*, 1993). Geyer and Schönherr (1990), however, showed that the barrier properties of isolated *Citrus aurantium* cuticles were strongly disturbed after enzymatic isolation. Water permeability decreased successively by a factor of up to 2.8 during a storing period lasting more than one year. The authors assumed that this was due to a "healing of defects" in the transport barrier of the cuticles generated by the isolation procedure. Since the permeation of water through cuticles cannot be ascribed exclusively to one of the two proposed pathways but probably involves both the lipohilic and the hydrophilic route (Schreiber *et al.*, 2001), the actual location and mode of action of such presumed defects remains unclear.

After isolation, cuticles are usually kept under variable environmental conditions for arbitrary periods of time before use. Cuticles are either stored dry (e.g. Coret and Chamel, 1993; Buchholz *et al.,* 1998; Schreiber *et al.*, 2001) or immersed in water (Orgell, 1955; Tyree *et al.,* 1990a). Storage times may range from "at least four weeks" (Baur *et al.*, 1999b) to "at least three months" without further details on exact duration (Buchholz *et al.*, 1998, Niederl *et al.*, 1998). In other studies no storage time was mentioned (Schönherr 1976a,b; Heredia and Benavente, 1991; Knoche *et al.*, 2000; Riederer, 2006) or it was not specified (Shafer and Bukovac, 1987; Schreiber *et al.*, 2001, Schönherr, 2001). Cuticles were stored at room temperature (e.g. Shafer and Bukovac, 1987; Knoche and Bukovac, 1993; Kirsch *et al.*, 1997) or refrigerated (e.g. Yamada *et al.*, 1964; Schönherr *et al.*, 2000, 2001).

Moreover, it was shown that cuticles inevitably adsorb significant amounts of lipophilic material originating from the leaf cell slurry during the isolation process (Schönherr and Riederer, 1986), and it cannot be a priori ensured that this might not additionally affect their barrier properties.

Besides the risk of altering the barrier properties of cuticles during isolation, the use of isolated cuticles for mechanistic penetration studies generally carries the inherent danger of obtaining biased results. This is due to the fact, that only astomatous cuticles of a few species can be isolated enzymatically, excluding most species of economic importance, such as most Gramineae species (Kirkwood, 1999). Moreover, trichomes which are present on the leaf surface of many species may affect the isolation procedure (Schönherr and Schreiber, 2004).

2. Current Evidence for a Different Size Selectivity of Cuticles for Hydrophilic and Lipophilic Solutes

One of the main arguments for the existence of two physically distinct cuticular pathways for hydrophilic and lipophilic solutes rests on the assumption of different size selectivities. Apart from the question of whether water clusters absorbed in the CP could create a pathway with such different size relations, it is necessary to check whether the conclusion that cuticles have different size selectivities for hydrophilics and lipophilics is justified.

There are two studies which deal with the effects of molecular size on penetration rates of both substance groups. In the paper by Popp *et al.* (2005) both groups were directly compared using cuticles of the same species. Schönherr and Schreiber (2004), however, compared their data obtained with ions with the data of lipophilics reported in a previous study (Buchholz *et al.*, 1998), and it is noteworthy that in both studies cuticles from two different poplar species were used. Apart from that, the size of the permeating molecules was expressed in different units. Bucholz *et al.* (1998) used molecular volume, whereas Schönherr and Schreiber (2004) used molecular mass. To

be able to compare their results with those of Buchholz *et al.* (1998), Schönherr and Schreiber (2004) used the equation:

$$\text{molecular mass} = 65.2 + 0.954 \times \text{molar volume} \quad (r^2 = 0.91) \qquad (2)$$

but it is unclear how this relationship was derived. Linear regression with the data (Table 1) published by Popp *et al.* (2005) gives:

$$\text{molecular mass} = -5.2 + 1.45 \times \text{molar volume} \quad (r^2 = 0.97) \qquad (3)$$

In Figure 2 it is shown how different ways to convert data related to molecule size affect the apparent size selectivities (= slopes of the graphs) of the different compounds and cuticles from different species. In the study of Popp *et al.* (2005), which is the only one in which both lipophilic and hydrophilic molecules were contrasted with the same species and under identical conditions, the hydrophilic path was more size selective (=steeper slope) than the lipophilic path. Analysis of the data of Buchholz *et al.* (1998) shows that the apparent size selectivity of the lipophilic path strongly depends on the equation used to convert molecular volume into molecular mass. Equation 2 from Schönherr and Schreiber (2004) yields a much higher size selectivity than equation 3 based on Popp *et al.* (2005). The original data published by Schönherr and Schreiber (2004) indicate a very low size selectivity of the hydrophilic path (Figure 2, line 3a). They studied the cuticular penetration of di-anionic Ca-salts and used the formula mass of the entire salt to calculate size selectivity. Since salt solutions contain dissociated ions, a calculation of size selectivity based on the mass of the individual ions could be more reasonable. A calculation using the molecular mass of the anion yields a considerably higher size selectivity, which is almost coincident with the size selectivity of the lipophilic path reported by Popp *et al.* (2005).

This comparison and re-evaluation of available data on size selectivity of the cuticle shows that there is no unambiguous evidence for a difference between hydrophilic and lipophilic solutes. Moreover, it is questionable whether this parameter enables a truthful evaluation of the physical processes, since it uses linear regression, which implies that any molecule, no matter how large it is, can still penetrate the cuticle without any size exclusion limit. Moreover, size selectivity is only a relative measure, and it is not possible to directly translate a certain value of size selectivity into the size of the voids in the cuticle.

3. Size Exclusion Limits of Cuticular Penetration

The observed size exclusion of large hydrophilic solutes was interpreted as evidence for the existence of polar pores in plant cuticles (Schönherr, 1976a). This concept thus assumes that water clusters traversing the cuticle from the leaf surface to the epidermal cell wall have a finite cross sectional area which limits the size of a permeating molecule. Interestingly, it was never attempted to determine a corresponding size exclusion of lipophilic molecules. Against the background of the recent debate on the size relations of the cuticular pathway, in particular regarding the discussion about different size selectivities (see above), this is quite surprising. Sound size estimations of both pathways could be helpful in proving or disproving the hypothesis that hydrophilic molecules use a diffusion route that is physically distinct from the lipophilic path.

The tri-dimensional spongy network of hydrocarbons of the cutin matrix, in which the lipophilic solutes diffuse, is believed to act as a molecular sieve (Luque *et al.*, 1995a), and it can thus be expected that molecules too large to fit into the cuticular voids are excluded from this route. Evidence for a distinct size distribution of cuticular voids comes from X-ray diffraction studies, which indicated the existence of two major spacings of 0.45 and 1.0 nm width in tomato fruit cuticles (Luque *et al.*, 1995a). However, respective data on cuticles from other species are not available yet.

Published estimations of average sizes of polar pores show a fairly broad range (from 0.3 to 2.4 nm), and it is to date not clear whether this is due to the different methods employed or whether it reflects the existing variability between plant species. At first sight, the differences might appear small; however, they could have serious consequences for the barrier properties of leaf surfaces. A pore with 0.3 nm radius would exclude large molecules such as sucrose and chelated micronutrients, whereas pores having radii of more than 1 nm would still be permeable. Therefore, the methods employed by different authors deserve a critical appraisal.

The first estimations of the size of pores published by Schönherr (1976a) were derived from the measurement of water penetration through dewaxed cuticles. He calculated pore radii (r_P) from the ratio of osmotically-induced and diffusional water fluxes. The basic idea was that any osmotic, i.e. pressure driven water flow requires pores and that the value of the flow is proportional to r_P^4, whereas diffusion in pores is proportional to r_P^2.

The much higher values of up to 2.4 nm published by Eichert and Goldbach (2008) were estimated from measurements of the simultaneous penetration of small (urea, ammonium or nitrate) and large molecules (sucrose) into intact leaves. They used the ratio of penetration rates of the solutes in relation to the ratio of their bulk diffusion coefficients to calculate the size relations of the penetration pathway shared by both molecules. Popp *et al.* (2005) used a similar approach. However, in this article too little information is provided regarding the calculation method of pore size to allow for a critical evaluation of the results.

The significant discrepancy could at least partly be attributed to the assumption by Schönherr (1976a) that diffusion of water through cuticles is totally restricted to aqueous pores. Recent evidence suggests that water, a small, uncharged molecule, is not excluded from the lipohilic path (Schreiber, 2002), and some authors assume that this path is even the most (Schreiber *et al.*, 2001) or the only relevant route (Schönherr, 2000). Water movement in the lipophilic path would cause an overestimation of diffusional flow through the supposed polar pores. Hence, the ratio of osmotic to diffusional pore flow and consequently the corresponding r_P would be underestimated. Using the data presented by Schönherr (1976a) (Table 9, equation 13) the dependence of r_P on the actual share of the lipophilic pathway in total water penetration can be calculated. Figure 3 shows that the allowance for a contribution of the lipophilic pathway to water penetration results in pore radii which are substantially larger than the estimations published by Schönherr (1976a). Assuming a 50% share of the lipohilic path, for instance, results in $r_P = 0.73$ nm, and a 90% share yields $r_P = 1.77$ nm. If the contribution of the aqueous pores is actually negligible, as proposed by Schönherr (2000), i.e., the share of the lipophilic path in water movement approaches 100%, even higher values of r_P can be deduced.

4. Effect of Humidity

In some experiments, the permeability of cuticles or leaf surfaces was studied at 100% RH (Schönherr, 2000, 2001; Schönherr and Luber, 2001; Schönherr and Schreiber, 2004; Eichert and Goldbach, 2008). From a thermodynamic viewpoint, water-saturated air is in equilibrium with pure liquid water, and therefore the equilibrium concentration of any solution would be zero. It can be safely concluded that the solutions in the studies mentioned above never reached equilibrium with the ambient RH, because this would require the absorption of an infinite amount of atmospheric water. It is thus very likely that the final concentrations of the applied solutes were indeed not zero, but very low. However, the exact solute concentrations

and their time course in a solution exposed to 100% RH depends on many factors such as the initial concentration or the earlier drying of the solutions (Schönherr, 2000; Schönherr and Luber, 2001; Schönherr and Schreiber, 2004). Consequently, in experiments conducted at 100% RH the driving force for penetration cannot be determined, rendering a mechanistic analysis of the penetration process impossible.

It has been shown that the hydration of cuticles decreases with decreasing humidity (Chamel *et al.,* 1991, 1992; Luque *et al.*, 1995b), and it was concluded that this reduces the permeability of the hydrophilic route (Schönherr, 2000). Simultaneously, decreasing humidities cause a drastic increase in the equilibrium concentration of foliar-applied solutes (unless the RH drops below the ERH of the respective compound, causing the solution to dry out). Both effects have an opposite effect on penetration rates, and any observed humidity effect on penetration rates thus cannot be directly ascribed to a change in the barrier properties of the cuticle.

Due to the limited knowledge of the relationship between RH and equilibrium solute concentrations, the interactions between humidity and cuticular permeability have so far been treated rather descriptively. However, models developed in aerosol science can be used to calculate equilibrium concentrations at any given RH. This is exemplified in Figure 4 for some N-salts. The maximum concentrations reached at the DRH of the respective salts, which are independent of the initial concentration of the solute, range from >6 to >25 moles kg^{-1} water. It can also be seen that the maximum salt concentration at DRH is not directly related to DRH; in other words, salts with a low DRH can but do not necessarily have a high saturation concentration. Moreover, it is remarkable that, for most of the salts, the decrease of concentration with increasing RH is almost linear. This can be utilised for a simple estimation of salt concentrations at any RH without the need for complex calculations.

Using this approach for an estimation of the RH-dependent equilibrium concentrations of salts, the existing publications on RH effects on the cuticular penetration of salts can be re-evaluated. Thereby, the effects of RH on the driving force (solute concentration on the leaf surface) can be separated from its effects on the permeability of the cuticle. This is demonstrated in Figure 5, where the rate constants of diffusion of Ca- and K-salts through isolated pear cuticles reported in different studies (Schönherr, 2000, 2001; Schönherr and Luber, 2001) were corrected for their respective humidity dependent equilibrium concentrations. When the RH was increased from 50% to 100% (a humidity of 99% was actually used for calculation of solute concentrations, because the solutions probably did not reach equilibrium during the experiments, see above), measured absolute rate constants increased in a linear fashion by factors of 2.9 to 3.6. If however the values are corrected for the associated decrease in driving force by dividing the rate constants by the corresponding equilibrium concentrations, an increase by factors of 114 to 142 is obtained (Figure 5). This is in line with reports on the hydration behaviour of cuticles indicating that the water content of a cuticle is not proportional to air humidity but increases steeply if humidity approaches 100% (Chamel *et al.*, 1991; Luque *et al.*, 1995b).

Decreasing RH from nearly 100% to 50% reduced the permeability to less than 1% of its initial value at full hydration (Figure 5), which indicates that the hydrophilic cuticular penetration path is possibly far more dependent on ambient humidity than previously assumed. The relatively high cuticular penetration rates of ions measured at low humidity are thus rather the consequence of the high solute concentrations on the leaf surface prevailing under such conditions, which can reach values of >10 M (Figure 5), rather than of a high permeability of the cuticle.

5. Possible Bias caused by Additives

The hypothesis of polar pores was stimulated by the feeling that penetration rates of ions, which have a low solubility in the cuticle, were higher than what could be expected from the laws of penetration established for lipophilic solutes (Schönherr, 2000). However, this rather subjective conclusion was never experimentally verified.

Interestingly, almost none of the papers aimed at characterising polar pores actually studied the pure intrinsic permeability of the cuticle. Instead, penetration was usually measured using surface active ingredients, which potentially alter the barrier properties of the cuticle. Schönherr, Schreiber and co-workers (e.g., Schönherr, 2001, 2002; Schönherr and Schreiber, 2004; Schlegel *et al.*, 2005) generally used Glucopon 215 CS UP (a C8/10-polyglucoside) or Genapol C-100 (a polydisperse ethoxylated alcohol), although Baur (1999) had shown that Genapol C-100 is an accelerator-type adjuvant, which may artificially increase the permeability of the cuticle for polar compounds. It was shown that penetration rates of ions can be substantially increased by these additives by factors greater than 6.5 (Schönherr, 2000). Unfortunately, data on the mode of action of Glucopon 215 CS UP as an adjuvant are not available. However, it was shown that this ingredient penetrated plant cuticles very rapidly (Bai *et al.*, 2008), indicating that its activity is not restricted to the leaf surface.

Shi *et al.* (2005) proposed that ethoxylated alcohols sorbed in the cuticle might even create an artificial polar pathway. If the additives used in previous studies had a similar accelerating effect their addition may strongly compromise the significance of the conclusions drawn regarding the nature of the penetration pathways of ions, because accelerators might have created artificial polar pathways which are absent in native cuticles. The standard use of adjuvants could also have prevented a significant effect of additional accelerators, which was stated as argument for the existence of polar pores (Schreiber, 2005). Therefore, future research aimed at elucidating native penetration pathways in cuticles should avoid the standard use of additives.

6. Conclusions on Diffusion Pathways of Hydrophilic Solutes across Cuticles

According to the concept of polar pores, lipohilics and hydrophilics diffuse in physically distinct routes. It is indeed very likely that the polysaccharide strands in the CL provide a penetration route which is accessible to and preferentially sorbs polar and ionic compounds (Hoch, 1979; Jeffree, 2006; Riederer, 2006), whereas it should more or less exclude lipophilics, which prefer the surrounding lipophilic cutin matrix. However, corresponding spatially separated paths in the overlying CP, which is free from carbohydrates (Jeffree, 1996), are less evident. Such a separate path for hydrophilics in the CP could be envisaged as clusters of water molecules sorbed in the cuticle bridging the gap between polysaccharide stands of the CL and the leaf surface.

Assuming that water absorbed in the CP, even if it is clustered, must still fit into the void spaces within the three-dimensional cutin network, the effect of solute size on penetration rates should be the same, whether it is lipophilic or hydrophilic. Differences in penetration rates between both types of molecules could then only be caused by the different physicochemical interactions between the molecules and the cuticular components due to charge or polarity. In other words, no differences in size selectivities should exist between lipophilic and hydrophilic compounds. This conclusion is preliminarily supported by the revision of available data on size selectivities of diffusion across plant cuticles showing that differences for lipophilic and hydrophilic molecules are not evident. Considering the scarceness and ambiguity of the available data, the effects of temperature, plasticisers and wax extraction on penetration of ionic

or polar molecules, which were cited as additional evidence for the existence of two pathways (Schreiber, 2005), certainly require further experimental studies.

The solution-diffusion model, which predicts the permeability of a lipophilic molecule from its solubility and mobility in the cuticle, is to date believed not to be applicable to hydrophilic molecules (Schreiber *et al.,* 1996b; Schreiber, 2006). If size selectivity, which is an indirect measure of the mobility of a molecule in the cuticle, turns out to be actually independent of the degree of lipophilicity or hydrophilicity of a penetrating molecule, this could open up the possibility to expand the existing solution-diffusion model into a unified model for uncharged solutes (ions, which interact with the fixed charges in the cuticle, could require a more complex model). In such a unified model, the effect of water sorption by the cuticle on uptake rates of hydrophilics, which was so far regarded as a process interfering with the assumptions of the solution-diffusion model, could be incorporated by its effect on the solubility of hydrophilic solutes in the cuticle.

VI. EFFECTS OF STOMATA ON FOLIAR PENETRATION OF HYDROPHILIC SOLUTES

A. Hypotheses

There is no doubt that lowering surface tension below a certain critical level can promote stomatal infiltration by dynamic mass flow of foliar applied solutions (Stevens 1993; Zabkiewicz *et al.*, 1993). This was convincingly demonstrated by Field and Bishop (1988) who studied the effect of the organosilicon surfactant, Silwet L77, on foliar uptake. Using fluorescein as a tracer, they found that this additive in concentrations of 5 g L^{-1} promoted a complete and immediate spreading of the foliar-applied solution on the surface of *Lolium perenne* and penetration of nearly all stomata present on the leaf surface.

However, in many other studies, in which no surfactants were used, stomata also contributed to the process of foliar penetration. At the latest, since the study of Schönherr and Bukovac (1972) indicating that infiltration of stomata is physically impossible, these observations raised serious discussions on the underlying mechanisms. Some researches assumed that the cuticle was more permeable over guard cells than over other epidermal cells (Schönherr and Bukovac, 1978). Others hypothesised that penetration of solutes occurred directly through the stomatal pores, but not via dynamic mass flow. Middleton and Sanderson (1965) already discussed "the possibility of surface migration of the solute through films of moisture on the leaf surface which might be connected to similar films on the interior by stomatal pores". Later this hypothesis was adapted by Burkhardt (1994), who suggested that thin water films present on the surfaces of leaves might enter stomatal pores. More recent studies with fluorescent tracers gave strong support for the idea that penetration of solutes via stomata can occur by diffusion along the pore surfaces without infiltration (Eichert 2001; Eichert *et al.*, 2008).

B. Model Systems and Methods

Isolated cuticles are inappropriate model systems for studying the effect of stomata on foliar penetration, since the barrier function of stomata against infiltration rests on the integrity of the unique architecture of guard cells (Schönherr and Bukovac, 1972). During the isolation process, cuticles are separated from the underlying epidermal cells, including the guard cells. Hence, isolated stomatous cuticles have physical holes with an undefined geometry, the barrier function of stomata being most probably lost after

isolation. Therefore, most researchers used either detached leaves or leaf discs in their laboratory studies.

Quantitative methods, i.e., measuring penetration rates into leaves, enable only indirect estimations of the relative contribution of the stomatal and the cuticular pathway, discrimination between both penetration mechanisms remaining difficult if not impossible. Therefore, imaging methods, which are qualitative rather than quantitative, were employed to assess the role of stomata.

The basic and challenging principle of imaging foliar uptake is to catch the penetrating solute "red-handed", i.e., while it is still located in its penetration pathway within the leaf surface. This requires great diligence to differentiate the solute inside its pathway from those portions which are either still outside the leaf, i.e., adsorbed to the outermost surface, or already inside the leaf interior, i.e., inside epidermal cells. Furthermore, it has to be assured that the detection method enables an unbiased reproduction of the processes inside the leaf surface, a point which deserves closer attention.

C. Imaging Foliar Penetration

Several authors used imaging to localise preferential points of penetration of externally-applied ionic or polar solutes. The earliest approaches date back to the late 1930′s as described in Section III. (e.g., Schumacher, 1936; Strugger, 1939). Later, several investigations were published in which penetration patterns were studied using either radio-labelled or fluorescent tracers or metal salt precipitates.

1. Autoradiographic Methods

In several studies foliar penetration was analysed using radio-labelled tracers. This method enabled both the precise quantification of penetration rates (Sargent and Blackman, 1962; Norris and Bukovac, 1969) and the imaging of the tracer distribution within the plant following foliar uptake. However, in most of the investigations, identification of preferential penetration loci was impossible due to the low resolution of the autoradiographic images (Barrier and Loomis, 1957; Bukovac and Wittwer, 1957; Koontz and Biddulph, 1957; Mäkelä, 1996). Franke (1964b) assessed the penetration of ^{14}C-labelled organic compounds (mainly carbohydrates and amino acids) through isolated epidermal strips of stomatous leaves of *Spinacia oleracea* or *Viola tricolor*. After application periods of less than 1 h, most of the penetrated solutes were detectable in the guard cell region and above the anticlinal walls of epidermal cells. However, spatial resolution was still too low to localise the substances more precisely. Besides the limited special resolution autoradiographic methods require laborious preparation steps (L'Annunziata, 1979) as well as long exposure times of several weeks (Franke, 1964a; Wehtje *et al.*, 2007), which is probably the reason why other methods were preferred by most researchers.

2. $HgCl_2$ Precipitation – Significance of "Ectodesmata"

"Ectodesmata" were initially regarded as protoplasmatic strands interfusing the cuticle (Schumacher and Lambertz, 1956; Franke, 1960) and were thought to be involved in foliar absorption and excretion (Franke, 1961, 1964b, 1967). They were visualised by a rather complex procedure involving incubation of leaves in Gilson solution (a mixture of ethanol, formic acid, oxalic acid and formalin saturated with $HgCl_2$) for several hours, separation of the epidermis from the underlying tissue,

washing in 30% ethanol, treatment with KI solution and staining with pyoktannin in 10% H_2SO_4. This procedure induced the occurrence of precipitates in the cuticles, which were particularly formed over anticlinal epidermal cell walls and guard cells (Franke, 1964b, Schönherr and Bukovac, 1970a).

Later it was shown that the final precipitates consisted of elemental Hg (Schönherr and Bukovac, 1970b) and that their distribution was not related to epidermal cell structures, but that it indicated preferential $HgCl_2$ penetration pathways in the cuticle (Schönherr and Bukovac, 1970a). As pointed out by Schönherr (2006), $HgCl_2$ exists in an aqueous solution mainly as an uncharged molecule and thus it probably penetrates the cuticle by the lipophilic path. Schönherr (2006) proposed that the Hg precipitates mark sites which are rich in non-ionic functional groups, such as aldehyde and phenolic hydroxyl groups, and not structures associated with the polar path. Moreover, as already shown by Franke (1964c), the formation of Hg precipitates was affected by the internal concentrations of reducing agents, such as ascorbic acid. Consequently the characteristic pattern of "ectodesmata" does not stringently indicate preferential pathways but could probably be caused by the inhomogeneous distribution of reducing substances in the leaves.

3. Fluorescent, Molecular Tracers

Strugger (1939) used the cationic fluorescent dye, berberine sulphate, to trace the transpiration stream in *Helxine soleirolii* Req. plants. In his experiments, he usually introduced the dye via cut stems and stem incisions and followed the distribution within the vascular system. He observed that within seconds the dye appeared in the leaves, staining first the membranes of the vascular system and later the membranes of mesophyll cells. The dye was also visible inside the stomatal pores and Strugger concluded that the cuticular ledges were stained. Intensity and speed of staining was increased when stomata were opened by the effect of light. In other studies it was also observed that berberine fed by the transpiration stream was detectable inside stomatal pores (Bauer, 1953; Maier-Maercker, 1979b), but foliar penetration was not tested by these authors.

Strugger (1939) also published the results of one trial in which he immersed leaves in a berberine solution and directly tested whether the dye penetrated the leaf surface. He observed a patchy staining of some stomatal cuticular ledges, the cuticle above the anticlinal epidermal cell walls and trichomes, but foliar penetration was not closely related to stomatal aperture. He speculated about the existence of pores located in the cuticular ledges which allow the passage of berberine.

To test this hypothesis, he developed a method in which the leaf surface was covered with gelatine containing 0.9% glucose and 0.1 KCNS, which forms a fluorescent precipitate with berberine. Within minutes, crystals were formed in the gelatine over stomata and the base of trichomes. In his interpretation, the osmotic gradient between the leaf surface and the gelatine cover induced a flow of water and berberine. The movement of berberine should indicate that it was not physically or chemically bound by the cuticular ledges and that the dye should move inside these structures in pores. From the properties of berberine, it can be concluded, that according to this hypothesis, the putative pores should be large enough to permit the passage of cationic molecules with a molecular mass of 336.4 g mol^{-1}.

Schönherr and Bukovac (1978) interpreted Strugger's observations as evidence that cuticular ledges are preferential points of penetration of foliar-applied solutes. Furthermore, the studies showing that more solutes accumulated in the vicinity of guard cells under conditions inducing high transpiration rates, were cited as proof that the

permeability of guard cell ledges is increased when stomata are open (Schönherr and Bukovac, 1978; Schönherr, 2006). Since the aperture of stomata affected the intensity of staining only in Strugger's transpiration experiments, but not in foliar uptake trials, and considering the fact that in the other reports foliar penetration was not examined, such conclusion on the permeability of guard cell ledges seems questionable

However, the accumulation of the dye during transpiration and its subsequent remobilisation by an osmotic gradient neither really proves that the dye was actually located in pores nor contradicts the assumption that it was simply deposited at the places where water was transpired. It is thus not clear whether the berberine molecules entered these structures, and if so by which pathway, or whether they were merely superficially absorbed. Berberine is widely used for the staining of lipophilic domains such as Casparian bands of roots and suberinised aerial plant parts (Brundrett *et al.*, 1988). It is therefore very likely that the preferential staining of cuticular ledges could only indicate a high density of suitable absorption sites, as already denoted by Maerker (1965). Furthermore, cuticular ledges are known to be also heavily stained by other lipohilic stains such as styryl dyes (Meckel *et al.*, 2007) and Nile red (Li *et al.*, 2007). The penetration of a lipophilic dye would certainly not require a hydrophilic pathway, thereby leading to the conclusion that berberine staining of cuticular ledges may not be suitable to prove the existence of polar pores. Finally it has to be considered that the outer cuticular ledges of stomata are located well above the stomatal throat, and that they usually form the outermost part of the guard cells, so that any compound adsorbed by this structure has still not entered the leaf interior. Even if stomatal ledges served as preferential points of entry of externally-applied solutes, this would still not answer the question of which pathway they used to migrate further into the leaf.

Dybing and Currier (1961) studied the foliar penetration of an anionic fluorescent dye (Na-3-hydroxy-5,8,10-pyrenetrisulfonate) into detached leaves of *Zebrina pendula*, *Pyrus communis*, and *Prunus armeniaca*, with or without the addition of different concentrations of surfactants. The dye entered the leaves via stomata, and penetration rates were higher when stomata were open. Stomatal penetration was not uniform but showed a patchy pattern, i.e. in some regions of the leaf many stomata were penetrated whilst in others the dye did not enter at all. The amount of fluorescent dye that penetrated the leaves increased with increasing surfactant concentration. Interestingly, stomatal penetration was also detectable in the absence of any additive.

Eichert *et al.* (1998) investigated the penetration of fluorescein across epidermal strips and intact leaf surfaces of *Allium porrum* without the addition of surfactants. They reported that the penetration rate of the dye was 30 times higher with open than with closed stomata. When epidermal strips were viewed from the inner side, some of the stomatal pores appeared to be filled with deposits of concentrated dye. The authors concluded that penetration of the dye took place directly via the pores without the involvement of the cuticle.

Eichert and Burkhardt (2001) introduced a new model system for the visualisation of penetration patterns of ions (anionic fluorescein or Fe-salts) based on epidermal strips. Ionic solutes applied to the outer side of the epidermis were immobilised at the inner side immediately after penetration by means of a moist ion exchanger membrane. Using this method, it was shown that in leaves of *Allium porrum* and *Sedum telephium* penetrable stomata were very heterogeneously distributed over the leaf surface. The percentage of penetrated stomata was usually below 10% of all stomata in contact with the externally-applied solution. Using the method of Eichert and Burkhardt (2001), Eichert (2001) observed that occasionally fluorescent dye applied to the outer side of

epidermal strips appeared at the inner side within a few minutes. He concluded that penetration occurred either by mass flow of the solution or by fast diffusion.

Eichert and Burkhardt (2001) proposed that active, penetrable stomata differed from inactive ones in terms of the wettability of the cuticle surrounding their guard cells. They suggested that hygroscopic substances present at the surface of stomatal pores, such as deposited atmospheric aerosols, microorganisms or salts delivered by the transpiration steam ascending the pore, could be responsible for the observed loss of their barrier function. Water absorbed to these hygroscopic modifications should serve as a trans-stomatal liquid connection in which solutes can penetrate the leaf surface. This conclusion was recently supported by the observation that about 60% of stomata remained activated during consecutive wetting and drying of foliar-applied fluorescein solutions (Eichert *et al.*, 2008).

4. Precipitation Techniques

Hoch (1979) was among the first to trace the transcuticular pathway by the insoluble reaction products of two soluble substances diffusing from opposite sides of isolated cuticles. He observed that the location and concentration of precipitates was dependent on both the concentrations of the chemicals and the timing of the application of the substances on either side of the cuticle. Different patterns were recorded when application of the chemicals on both sides started simultaneously as opposed to one side being treated before the other.

More recently, Schreiber *et al.* (2006) studied the penetration of Ag^+ and Cl^- across isolated cuticles. Ag^+ was applied as soluble $AgNO_3$ on the outer side of the cuticle, while the inner side was exposed to a NaCl solution. They detected precipitates of AgCl on the outer surface of the cuticles.

The AgCl precipitation technique was also used to investigate foliar uptake into intact stomatous leaf surfaces. However, these studies differ from those carried out with isolated cuticles in one crucial aspect: with intact leaves, the precipitation of Ag^+ is induced by endogenous Cl^- ions, which are present native to the leaf tissue. Consequently, the actual Cl^- concentration is not under experimental control. Schlegel and Schönherr (2002) applied $AgNO_3$ together with a wetting agent (Glucopon CS UP) to intact stomatous leaves of *Vicia faba* and *Zea mays* for 12 h and observed silver precipitates "over and in stomata" and trichomes. Schlegel *et al.* (2005) applied $AgNO_3$ solutions to *Vicia faba* leaves for 1 h. The solutions contained a surfactant (Genapol C-200) and the authors could not rule out the possibility that this enabled infiltration of stomata. Using light microscopy and SEM they detected silver deposits on the surface of guard cells and around the bases of trichomes, which were identified as AgCl. Especially dense AgCl deposits were detected around stomata, but not at the cuticular ledges. As compared to the controls, both trichomes and guard cells appeared shrunken and disrupted, indicating that the Ag^+ ions were probably phyto-toxic. Schreiber (2005) published similar results concerning trials with $AgNO_3$ and *Vicia faba* leaves, but no experimental details were given. He also reported that AgCl deposits were located in and around stomata and at the bases of trichomes. These results were interpreted as evidence for preferential hydrophilic pathways in the cuticle surrounding guard cells, which could explain the dependence of uptake rates on the presence of stomata without the need of a direct involvement of the stomatal pore.

5. Imaging Foliar Penetration with Confocal Laser Scanning Microscopy

Eichert *et al.* (2008) studied the penetration of fluorescent water-suspended hydrophilic particles into leaves of *Vicia faba* at 100% RH. The use of suspended

particles instead of molecules made it possible to analyse the contribution of stomata to foliar penetration processes without the involvement of cuticular penetration. They demonstrated by CLSM that particles with a diameter of 43 nm entered stomata and migrated along the surface of the stomatal pores. Similar to the previous studies with fluorescein (Eichert *et al.*, 1998; Eichert and Burkhardt, 2001) only a small percentage of stomata were penetrated. Stomatal penetration was size selective, since particles with a diameter of 1 µm were excluded while the 43 nm particles were not. The authors reported that it took more than one day before substantial amounts of the 43 nm particles had entered the leaves. The size exclusion of the large particles together with the slow penetration of the small particles led Eichert *et al.* (2008) to conclude that penetration took place by diffusion in a liquid phase adsorbed to the walls of stomatal pores and not by mass flow of the suspension through stomata.

The use of particle suspensions instead of dissolved molecules solved the problem of how to distinguish cuticular from stomatal penetration. However, it posed a new methodical problem. Suspended particles tend to coagulate, especially if the particle concentration increases by evaporation of the solvent. Therefore, Eichert *et al.* (2008) had to conduct their experiments in water-saturated air, which possibly favoured condensation processes inside the stomatal pores or microbial growth and thus stomatal penetration. Further research is needed to elucidate both the physical basis of stomatal uptake and the importance of this pathway at lower RH.

D. Indirect Quantitative Methods

Since the discovery of the specific role of stomata in foliar penetration, there have been attempts to quantify their contribution in contrast to uptake through the rest of the cuticle. The comparison of stomatous and astomatous isolated cuticles (Kannan, 1969; Norris and Bukovac, 1969) or leaves (King and Radosevich, 1979; Knoche and Bukovac, 1992) indicated higher penetration rates in the presence of stomata, but this surplus of penetration rates could not be simply ascribed to stomatal uptake, because permeability of the cuticle could also differ between both leaf sides (Bukovac, 1976), as it was recently demonstrated by Santrucek *et al.* (2004) for the penetration of water.

Other studies aimed at selectively blocking either the stomatal or the cuticular penetration pathway. It was assumed that after closure, stomata could no longer contribute to foliar uptake (Sargent and Blackman, 1962). In another study, there was an attempt to block the cuticular pathway by coating the cuticle with gold particles (Reed and Tukey, 1982). None of these methods, however, proved successful. In particular, the argument that closure of stomata would totally disable the stomatal path needs further discussion. It is a matter of definition at which aperture stomata can be regarded as closed. Due to the limited spatial resolution of conventional light microscopy, a stoma having an aperture below 0.5 µm is usually regarded as closed. However, whether stomata are ever completely closed, i.e., the pore can be sealed airtight, is not presently known, and measurement of transpiration rates suggest that it probably does not occur (Kerstiens, 1996b). According to the results of Eichert *et al.* (2008), even small apertures below 0.5 µm could still enable the penetration of externally-applied solutions though the pores. Therefore, induction of stomatal closure does not safely block this pathway. Moreover, stomata usually show a patchiness of apertures (Eckstein *et al.*, 1996), so that some stomatal pores might still be considerably opened whereas the majority are closed. Hence, penetration of solutes through leaf surfaces with stomata presumed to be closed does not imply that the stomatal pathway is not involved in the foliar uptake process.

Eichert and Goldbach (2008) studied the penetration of small nitrogen compounds together with sucrose through stomatous leaf surfaces of diverse species and estimated the size relations of the underlying penetration pathways, assuming that penetration took place in pores with a circular shape. According to their calculations in leaves of *Coffea arabica*, *Prunus cerasus* and *Vicia faba*, equivalent pore radii were larger than 20 nm. The authors concluded that a pathway with such dimensions could not be located inside the cuticle, since previous estimations of the size relations of the hydrophilic path indicated radii which were one to two orders of magnitude lower. They postulated that this pathway was localised inside the stomata, which was later corroborated by the demonstration of particle penetration along the surfaces of stomatal pores (Eichert *et al.*, 2008).

Furthermore, Eichert and Goldbach (2008) found evidence for a pathway which excluded sucrose molecules due to their size, which they ascribed to small polar cuticular pores with radii <0.5 nm. In stomatous leaf surfaces they calculated the contribution of larger pores (radius >0.5 nm) to overall foliar uptake to range from 66 % in *Vicia faba* leaves to 89% in *Populus x canadensis*. In *Vicia faba* leaves with open stomata, the share of large pores was significantly higher than in leaves with closed stomata, which supports the conclusion that the stomatal pathway was involved. However, it was not possible to further quantify the exact share of stomatal uptake, because an unknown fraction of total uptake via pores with radii > 0.5 nm also occurred via the cuticle.

E. Critical Evaluation of the Current State of Research

1. Limitations and Drawbacks of Conventional Tracer Studies

Every experimental approach aimed at localising preferential points of entry of tracers using two dimensional top views of leaf surfaces suffers from a range of fundamental, mainly technical constraints, which seem to have been overlooked by most authors. These constraints, however, make any exact localisation of penetration paths impossible and thus any clear-cut conclusion obsolete. These problems are as follows:

- The documented distribution of penetrated foliar-applied substances in the leaf tissue does not necessarily reflect the point of entry. Penetrated substances usually move from the entry point by apoplastic diffusion or uptake into cells, which might lead to an enrichment of tracers in certain areas not related to the actual penetration process. An example may be the conclusion that guard cells are preferential sites of foliar uptake as observed in several studies.
- The resolution of the light microscope, which has been used in many investigations both with precipitates (Schlegel and Schönherr, 2002; Schlegel *et al.*, 2005) and fluorescent dyes (Strugger, 1939; Eichert *et al.*, 1998; Eichert and Burkhardt, 2001), is around 0.5 µm. This limited resolution together with the rather course optical signals of tracers (see below) makes it impossible to unequivocally assign the localisation of tracers relative to the fine micro-morphological structures of leaf surfaces.
- Two dimensional top views of leaf surfaces or cuticles do not allow for the exact spatialisation of tri-dimensional structures, because structures originating from different depths are projected into the two dimensional image producing identical indistinguishable signals in the projection plane. This was the main problem with the images published by Strugger (1939), where the low magnification additionally impeded clear interpretation of the dye distribution. It was thus impossible to distinguish whether the tracer was located in the cuticle, and if so at what depth, or whether it was only superficially adsorbed. The same applies to images showing fluorescent tracers (Eichert *et al.*, 1998) or AgCl precipitates in top views (Schlegel and Schönherr, 2002; Schlegel *et al.*, 2005). Cross sections could be used to map the migration of externally applied solutes in the third dimension, but sectioning always bears the risk of artefacts rising from spreading the solute under investigation across the cut surface. This is probably the reason why cross sectioning was hardly ever employed to image foliar uptake pathways.
- Tracers produce rather course signals. Fluorescent dyes tend to outshine their surrounding, which makes fluorescent spots appear disproportionally large while the surrounding region is obscured, thereby impeding their exact localisation. This problem was partially present in the study of Eichert *et al.* (1998), where accumulated fluorescein, although clearly visible in the centres of stomatal pores, could not be exactly localised with respect to guard cell morphology.
- Precipitates also tend to be rather course. Growth of precipitates is a continuous process which is not limited much by the surrounding solid matter. The forces exerted by growing salt crystals might even destroy concrete, so there is good reason to assume that precipitates growing in the cuticle will not necessarily reproduce the original size or shape of the pathways in which the ionic precursors of precipitates migrate, and might even affect the structural integrity of the cuticle. This conclusion is supported by Hoch (1979), who observed that growing Ag precipitates “ruptured the cuticle”.

- Detection of tracers requires them to be present in substantial amounts above their respective detection limit. Penetrating substances migrate in the cuticle as discrete molecules or ions, and the existing methods do not allow for the detection of single molecules. It is therefore very likely that any imaging method will preferentially and perhaps, exclusively detect places where there is a substantial accumulation of the substances, which is not necessarily identical to the places where they actually migrate. This can be illustrated by Franke's (1964a,b) observations that externally applied tracers accumulated in the guard cells. This finding does not allow a clear conclusion about where and how the tracers entered the leaf. Besides penetration of the external guard cell cuticle, as proposed by Franke (1964a,b), solutes may also have entered the guard cells after stomatal penetration, as was shown by Eichert (2001) for fluorescein. Precipitation techniques are likewise affected by this ambiguity. In the study of Schreiber *et al.* (2006), for instance, AgCl precipitates occurred at the outer cuticular surface, which only proves outward-directed penetration of the Cl^- ions, but it can neither be deduced via which pathway nor that Ag^+ entered the cuticle at all. Moreover, as indicated by the experiments of Hoch (1979), the location of precipitates depends strongly on the experimental conditions, especially on the concentrations of applied substances and the timing of application. Precipitation methods could thus preferentially indicate the locations where the two fronts of substances applied to opposite sides of the cuticle meet, the routes followed by the diffusing molecules before they encounter remaining unclear.
- The intensity or even the existence of an optical tracer signal can depend on other factors, which could lead to a misjudgement of concentrations of fluorescent molecules in plant tissues due to interactions with their microenvironment. Fluorescence intensity can be pH-dependent and modified by the adsorption to structures with distinct physico-chemical properties (e.g. lipophilicy or charge). Fluorescein fluorescence, for instance, is strongly increased in an alkaline environment (Martin and Lindqvist, 1975). Berberine shows a rather weak fluorescence in aqueous solution (Strugger, 1939). The fluorescence intensity is strongly increased by adsorption by suberized and lignified cell walls, and to a lesser degree by cell nuclei, cytoplasm and unmodified walls (Brundrett *et al.*, 1988). It is therefore possible that the staining pattern observed by Strugger (1939) was mainly the result of an increased fluorescence intensity of berberine induced by the presence of specific physicochemical structures, while berberine located in other structures was not detected.
- Precipitation of AgCl is likewise dependent on factors other than merely the permeability of the cuticle for the Ag^+ ions, because it requires the presence of Cl^- in the leaf tissue. Precipitation occurs whenever the product of the activities of Ag^+ and Cl^- exceeds the solubility product of AgCl. An inhomogeneous pattern of AgCl precipitates could therefore be the result of preferential foliar penetration pathways causing an inhomogeneous distribution of Ag^+, as assumed by many authors, or the consequence of an inhomogeneous distribution of Cl^- ions within the tissue, a factor which has been overlooked in previous studies. EDX analyses indicated that Cl^- concentrations in the guard cell region were significantly larger than in other parts of the epidermis (Schlegel *et al.*, 2005), which can be ascribed to the involvement of Cl^- in stomatal movement (Penny *et al.*, 1976; Hanstein and Felle, 2002). Moreover, experiments published by Höfler (1939) in which leaves *of Vicia faba* were perfused by $AgNO_3$ solutions showed that Ag^+ reduction occurred only in the guard cells but not in other epidermal cells. Hence, it is unknown whether the high density of AgCl precipitates around stomata as reported by, for example, Schlegel *et al.* (2005) really

indicates preferential penetration Ag^+ or is rather the consequence of the high local Cl^- concentrations or the high reducing capacity of guard cells.

To summarise these arguments, it becomes clear that virtually all previous studies aimed at visualising penetration pathways were not conclusive and that there is space left for controversy and conflicting interpretations. In particular, the principal question of whether a solute can penetrate stomata without any involvement of the cuticle cannot be fully answered by the existing methods. The main obstacles have to do with technical shortcomings such as the uncertainties concerning the significance of precipitate formation, the limited special resolution of light microscopy and the mapping of the three dimensional reality onto two dimensional images.

It is doubtful whether the main inherent problem of the AgCl precipitation technique, i.e. the uneven plant distribution of the counter ion Cl^- inducing the precipitation of externally applied Ag^+ ions can ever be solved. The spatial resolution of conventional light microscopy can be improved by electron microscopy, but the electron opaqueness of the specimens usually prevents the documentation of the processes underneath the leaf surface (SEM) or requires complex preparation steps, which involve the risk of artefacts (TEM). The third main obstacle, the lacking third dimension in conventional microscopy, can be overcome by the powerful technique of confocal laser scanning microscopy (CLSM), which has been recently been used to detect foliar penetration of nm-particles (Eichert *et al.*, 2008). This technique could prove to be a powerful and conclusive tool for the identification of penetration pathways of foliar-applied compounds.

2. Conclusions on the Involvement of Stomata in Foliar Penetration

To date, there seems to be no serious doubt that stomata play a significant role in foliar uptake of solutes. However, it is still a matter of debate by which mechanism(s) stomata facilitate the penetration process. The seminal paper of Schönherr and Bukovac (1972) has significantly contributed to our understanding of why stomata can't usually be penetrated by aqueous liquids and therefore terminated the discussion about spontaneous infiltration of foliar-applied solutions.

However, this study seems to have been over-interpreted in the past three decades, since the following aspects were not taken into account: First, Schönherr and Bukovac (1972) did not study penetration of solutes but of water as a solvent. Second, they considered infiltration as the only possible mechanism by which any non-gaseous substance can traverse a stomatal pore. Third, their argumentation rests on the assumption of an ideal stomatal pore, which is absolutely free from defects or contaminations. These interpretations finally led to the idea that stomatal pores, although they are holes in the leaf surface, can under no circumstances contribute to foliar penetration, which in turn implied the reverse conclusion that foliar uptake of any solute inevitably required penetration of the cuticle.

A range of hypotheses has been developed in the past to reconcile the apparently paradoxical promoting-effect of stomata on foliar uptake and the impossibility of their infiltration. At first, it was proposed that the cuticle in the guard cell region is rich in protoplasmatic "ectodesmata" serving as preferential penetration pathways. Later, these structures didn't turn out to be protoplasmatic nor did they indicate a preferential hydrophilic penetration route. Thereafter, it was hypothesized that the cuticular ledges of guard cells may serve as preferential sites of penetration and that the permeability of these structures increased with increasing stomatal aperture. However, the first assumption rests on observations with a fluorescent dye which probably preferentially stains specific lipophilic moieties, and the second assumption was never tested. Therefore, this hypothesis cannot at present be definitely evaluated. The same applies to studies in which the hypothesis of a higher permeability of the guard cell cuticle was tested using Ag^+ ions as tracers. It is to date not clear yet whether the preferential precipitation around guard cells truly supports this hypothesis or whether it is biased by an inhomogeneous Cl^- distribution or exclusive reducing capacity of the guard cells.

In contrast to these hypotheses, the proposed stomatal pathway located at the surface of stomatal pores does not require peculiar properties of the guard cell cuticle. Previous studies demonstrating such a pathway with molecular fluorescent dyes were as speculative and ambiguous as the studies aimed at the identification of preferential pathways in the guard cell cuticle. However, recent investigations using two independent methods, the calculation of size relations of the hydrophilic penetration pathway in stomatous leaf surfaces and the CLSM study of particle penetration, provided concurrent evidence for such a pathway.

VII. CONCLUSIONS AND IMPLICATIONS FOR FUTURE RESEARCH

The penetration and physiological effect of leaf-applied nutrient sprays involves a series of intricate mechanisms ranging from the mode of application, to the physical-chemical characteristics of the solution, the prevailing environmental conditions or the target plant species. The great complexity and multidisciplinary nature of the processes involved, hinders the development of strategies to optimise to efficiency of foliar sprays under different growing conditions and diverse plant species. Furthermore, the current

state of knowledge of many of the mechanisms involved is still factionary as described in Sections III. to VI.

Analysing the perspectives of foliar nutrient fertilisation stepwise, it can first be said that development of models to predict droplet size and spray performance after application will help improve leaf coverage, minimising spray drift and increasing the chance of foliar penetration under field conditions. Research on the effect of formulation additives will also ensure a better plant response provided that mechanisms of foliar uptake are first taken into consideration. Also in practical terms, research on appropriate spray timing and alternative application strategies, for example concerning optimal diurnal times and target tissues, will improve the reproducibility of the effect of foliar sprays for commercial use in different areas of the world.

The role of plant stress physiology concerning the response to leaf-applied nutrients should be elucidated, especially since foliar sprays are chiefly used to overcome nutrient and very often micronutrient deficiencies which often occur in arid and semi-arid areas in the presence of soils involving problems for plant growth (e.g., high pH, calcareous or saline soils).

Concerning the leaf surface, multiple factors relating to the behaviour and development of stomata, waxes and epidermal cells as described in Section IV. should be further investigated, since they will have a direct effect on the penetration mechanisms associated with one plant species grown under different conditions.

After analysing in detail the state-of-the-art on the penetration mechanisms of leaf-applied solutes, it can be concluded that more research efforts are required to clarify the mechanisms of cuticular and stomatal penetration. With regard to the penetration of water and solutes through the cuticle, there is a need to investigate the underlying mechanisms, avoiding the risk of increasing the complexity of the picture via addition of adjuvants that may affect the physical-chemical properties of the cuticle itself. The scene is so complex and heterogeneous that it is currently not possible to make a model to predict in spatial nano-terms the reaction of the existing lipophilic and hydrophilic functional groups in the cuticle to the penetrating solute.

Thereby, there is a need to apply less of a “black-and-white” approach to the problem of cuticular solute penetration, making use of recent advances in materials, aerosol and nanotechnology research. The research on isolated astomatous cuticles enabled modelling and understanding of many aspects of the penetration of leaf-applied chemicals. However, such simplified and artificial models may yield biased or/and oversimplified results, which may have little to do with the penetration mechanisms occurring in intact leaves and in the presence of functional stomata. The significance of the stomatal pathway and the related mechanisms should be further investigated with methodologies which avoid the pitfalls described in Section VI. In this regard, foliar sprays should target open stomata as a means to a priori maximise the chance of foliar penetration.

On the other hand, it should be possible to improve the performance of nutrient formulations using the models developed in the field of aerosol particle research. Progress in aerosol research has to date gained a detailed picture of the deliquescence behaviour of hygroscopic particles, enabling, for example, the prediction of exact equilibrium concentrations of solutions above the DRH (Pilinis *et al.*, 1989; Tang and Munkelwitz, 1993) as well as the deliquescence behaviour of supersaturated (Kelly and Wexler, 2006) and of mixed solids, i.e. systems containing more than one salt (Tang and Munkelwitz, 1993; Ansari and Pandis, 1999). Models have been developed that enable the calculation of solute activities in aqueous electrolyte solutions (Clegg *et al.*, 1992), the influence of mineral acids (Carslaw *et al.*, 1995, Massucci *et al.*, 1999,), the

deliquescence properties of and equilibrium concentrations in multi-salt and acid systems (Clegg *et al.*, 1998, 2001) as well as of systems containing organic acids (Clegg and Seinfield, 2006a,b). Since the thermodynamic processes governing aerosols also apply to bulk solutions, (Tang and Munkelwitz, 1993), including and applying the entire body of knowledge acquired in aerosol research foliar penetration studies could certainly contribute to an improved understanding of the underlying mechanisms.

Future basic research, conducted in a critical and holistic way, will help further elucidate the multiple processes involved in foliar penetration and the physiological effect of sprays in plants, closing the gap between the reality in the field, thereby contributing to an economically efficient and environmentally friendly use of foliar-applied sprays in agriculture.

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Figures

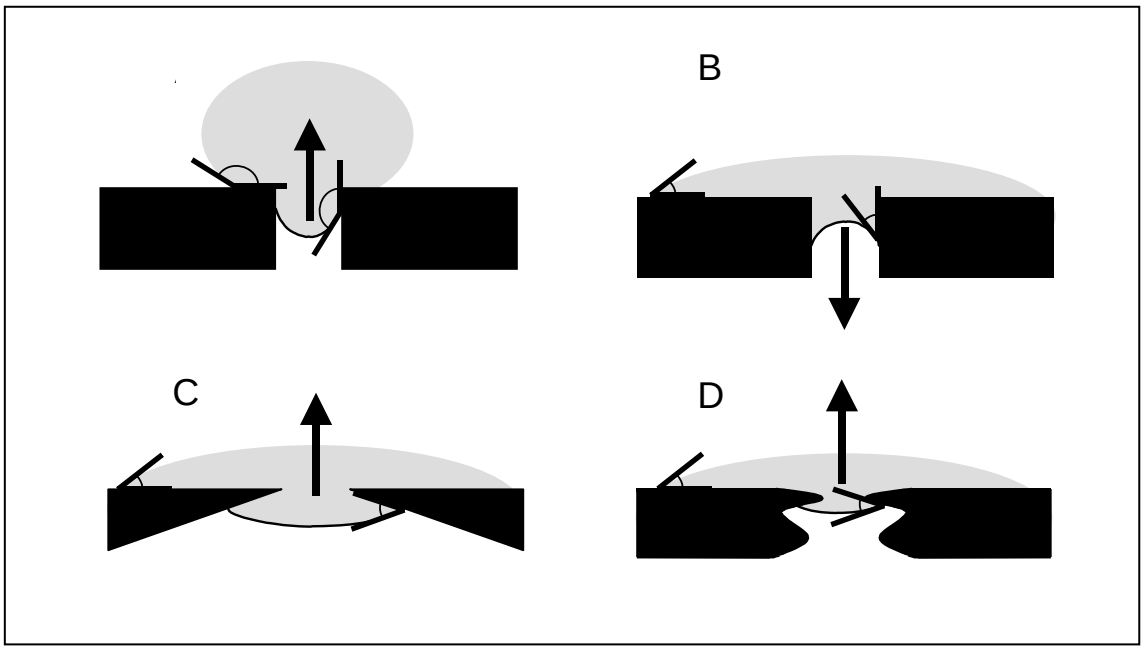

FIGURE 1. Simplified illustration of the barrier function of stomata against the infiltration of aqueous solutions. A droplet (grey) rests on a capillary. The liquid forms a contact angle θ with the capillary material. The situation immediately after the intrusion of the liquid into the capillary is depicted. The meniscus at the liquid frond generates a pressure difference. Spontaneous infiltration requires that the force (arrow) is directed inwards the capillary, a outwards directed force prevents infiltration. A: $\theta > 90°$, cylindrical capillary: no infiltration. B: $\theta < 90°$, cylindrical capillary: spontaneous infiltration. C: $\theta < 90°$, diverging capillary: no infiltration. D: schematic cross section of a stoma. Outer cuticular ledges form a diverging capillary preventing infiltration of liquids with $\theta < 90°$. Based on Adam (1948) and Schönherr and Schönherr and Bukovac (1972).

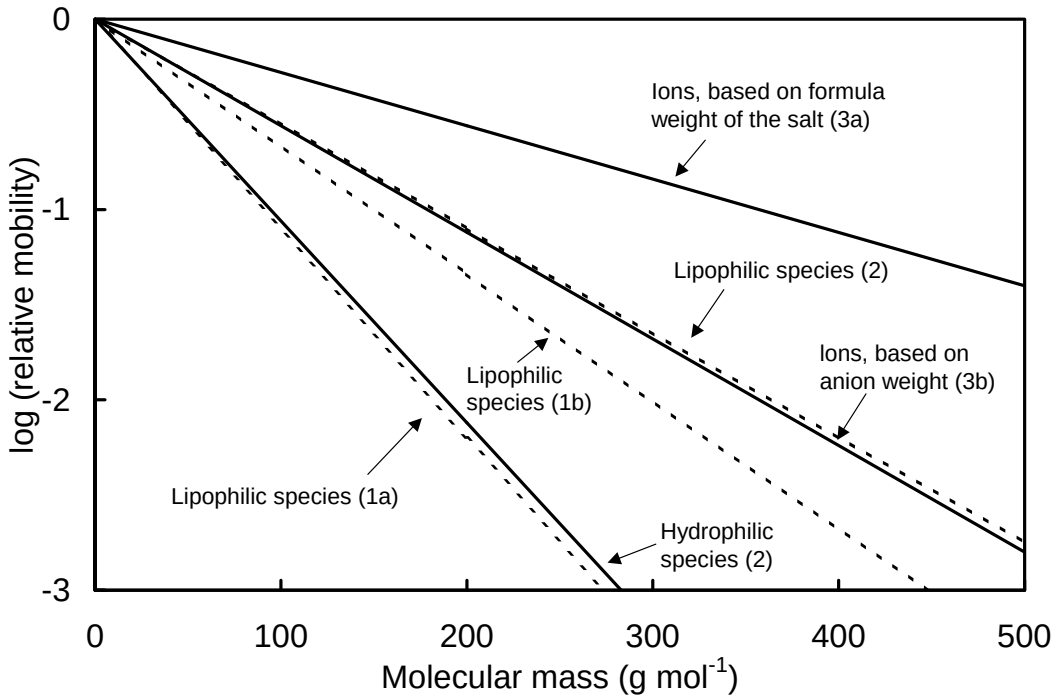

FIGURE 2. Correlations between the molecular mass of lipohilic (dashed lines) and hydrophilic solutes (solid lines) and their relative mobility across astomatous isolated cuticles of different plant species. Based on data from: (1) Buchholz et al. (1998) (*Populus alba*), (2) Popp et al. (2005) (*Hedera helix*) (3) : Schönherr and Schreiber (2004) (*Populus canescens*). For comparability, the mobility of a molecule with zero molecular weight was set to zero. Data from (1) were converted into molecular mass using: (1a) the conversion proposed by (3) or (1b) linear regression calculated from data given in (2). Data from (3) are presented as given by the authors (3a) and recalculated (3b) to show the dependence of mobility on the weight of the largest ion instead of the gross formula mass of the salt.

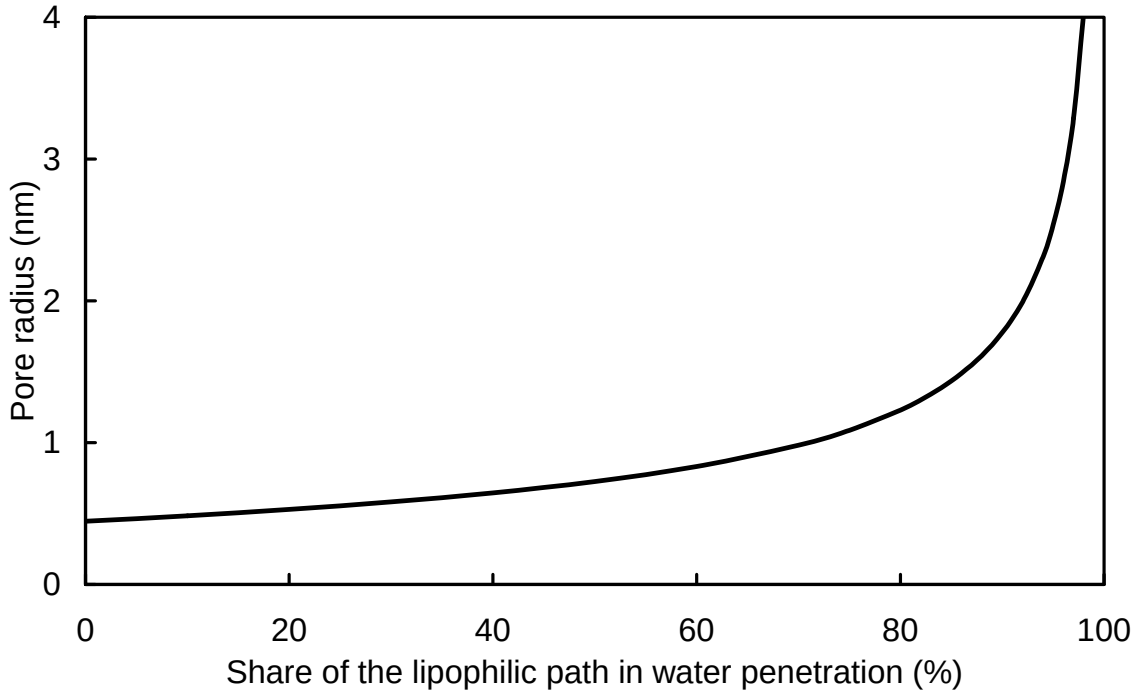

FIGURE 3. Dependence of values of pore radii on the actual share of water penetration across the lipophilic route across cuticles of *C. aurantium*. Data were taken from Table 9 and pore radii were calculated with Equation 13 given in Schönherr (1976a), assuming that the diffusional flow of water is composed of flow across both the lipophilic and the hydrophilic path. The radius of 0.45 nm at 0% share is the value reported by Schönherr (1976a)

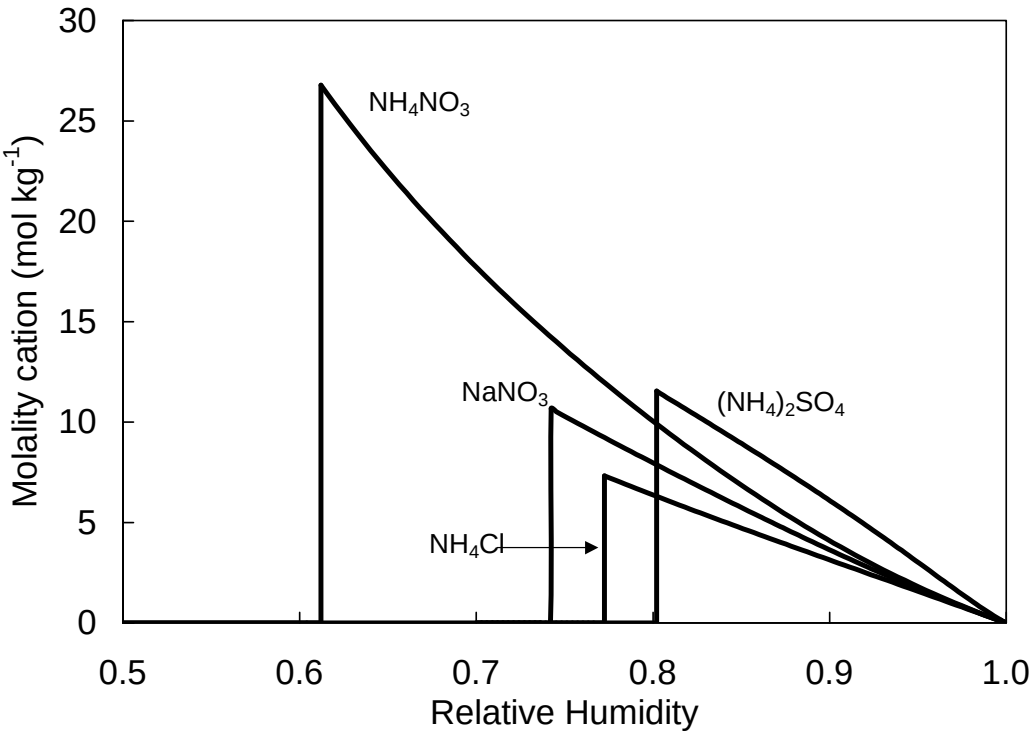

FIGURE 4. Equilibrium concentrations of some N-salt aerosols as effected by relative ambient air humidity. At the respective DRH of a salt a sudden transition between the solid and the dissolved state occurs. Data were calculated using the Extended Aerosol Inorganics Model of Clegg, Brimblecombe and Wexler (http://www.aim.env.uea.ac.uk/aim/aim.php) for a cation concentration of 1 mol m^{-3} air.

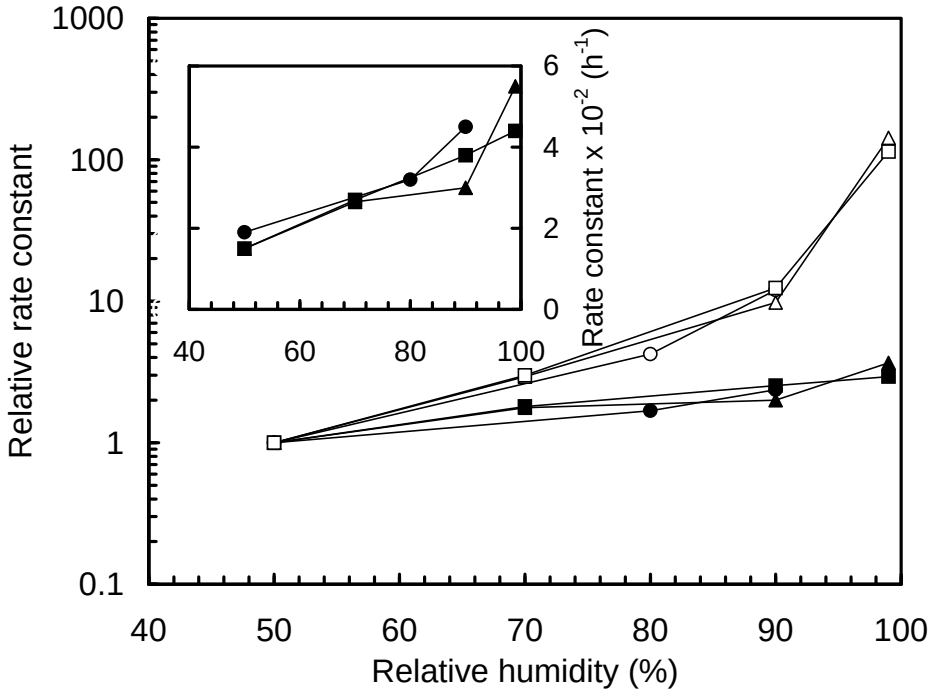

FIGURE 5. Rate constants of diffusion of Ca^{2+} and Rb^{+} across isolated astomatous pear cuticles as affected by relative air humidity. Note the logarithmic scale of the ordinate. Recalculated data from: Schönherr (2000), Ca^{2+}, triangles; Schönherr (2001), Ca^{2+}, squares; Schönherr and Luber (2001), Rb^{+}, circles. Values are expressed relative to the rate constant measured at 50% RH, which was set to 1. Filled symbols are based on the original data presented by the authors. Open symbols are recalculated values obtained by dividing the rate constants by the equilibrium concentration of the salt at the respective humidity. Equilibrium concentrations were calculated using tabulated solubility data assuming maximum concentrations at the deliquescence humidity of each salt, a linear decrease of concentration with increasing humidity, and a concentration of zero at 100% humidity. The insert shows the absolute values of rate constants as given be the authors.